

Identifying cellular kinases that regulate HSV-1 ICP0 activities and viral
replication.

By

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Abstract

Herpes simplex virus 1 (HSV-1) infects a significant number of people worldwide and is the number one cause of infectious blindness in western industrialized countries. HSV-1 causes a lifelong latent infection that can be reactivated by various stressors. HSV-1 E3 ubiquitin ligase phosphoprotein, infected cell protein 0 (ICP0), is a potent global transcriptional activator of genes and is required for productive viral replication and reactivation from latency. While the phosphorylation state of ICP0 is important for regulating many activities of ICP0, there is only a minimal amount known about which specific cellular kinases are important for modulating these activities. Studies with HSV-1 have been done with Roscovitine (Rosco), which inhibits CDK1, CDK2, CDK3, CDK5, CDK7, and CDK9 have shown that Rosco impairs HSV-1 replication, reactivation, the transactivation activity of ICP0, and it alters the posttranslational modification state of ICP0. It is not known which of these specific kinases are required for regulating these activities.

This thesis summarizes my studies to identify specific cellular kinases that are required for the transactivation activity of ICP0 and regulate its other key functions to promote efficient viral replication and reactivation from latency. To begin to elucidate which specific cellular kinases are important for the transactivation activity of ICP0, we developed a high throughput cell-based assay using HSV-1 GFP reporter viruses with small interfering (siRNA) in a 96-well plate format. siRNA library used in screens was against ~700 known and

putative cellular kinases, from which I identified several (~21) potential regulators of ICP0's ability to stimulate viral gene expression.

From the list of kinase candidates, we focused our studies on cyclin-dependent kinases (CDKs) 1, 2, 4 and 6, which are associated with the cell cycle and did not appear to have an inhibitory effect on ICP0 protein expression in siRNA-transfected cells. We decided to use specific CDK inhibitors to examine the role activities of specific CDKs play in modulating ICP0 function. Our data shows that inhibiting CDK1 impaired HSV-1 replication in an ICP0-dependent manner, while the replication of wild-type (WT) HSV-1 and an ICP0-null mutant was diminished when the CDK2 inhibitor was tested. We then examined the effect of these CDK inhibitors on viral protein expression in WT HSV-1 infected cells. While the CDK1 inhibitor reduced levels of UL42, an early protein; the CDK2 inhibitor had a much greater effect by decreasing or inhibiting UL42, ICP8, US11, and VP5 expression. None of the CDK inhibitors appeared to have an effect on ICP0 localization or ND10 disruption. Finally, using a quiescent infection model, we demonstrated that inhibitors of CDKs 1 and 2 repressed ICP0-induced reactivation of viral gene expression. These results strongly suggest that CDK1 regulates an aspect of ICP0's transactivation activity, which in turn enhances HSV-1 lytic infection and reactivation. While CDK2 also appears to regulate (at least in part) ICP0's ability to stimulate gene expression during lytic replication and reactivation, it also aids HSV-1 productive infection cell culture in an ICP0-independent fashion.

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Chapter 1: Introduction

1.1 Herpes simplex virus type 1 (HSV-1)

Human herpesvirus 1 or herpes simplex virus type 1 (HSV-1) is known to infect approximately 70-80% of the population worldwide [1]. The most common sign of infection in humans is a cold sore that can form around the mouth or lips, although the infection in some individuals is asymptomatic [1]. HSV-1 is also capable of infecting eyes and is the number one cause of infectious blindness in western industrialized countries [1]. HSV-1 can also be a cause of genital herpes and can cause encephalitis in immunocompromised individuals.

HSV-1 is an enveloped virion and contains a proteinaceous layer underneath the envelope known as the tegument, which surrounds an icosahedral nucleocapsid. The envelope of the virus, which is derived from the host cell membrane, contains glycoproteins that transverse the envelope and aid the virus in attaching and entering into the cell. The viral genome within the capsid is a large, linear double-stranded DNA genome that is approximately 152 kilobases [1]. HSV-1 infects only humans and is a member of alphaherpesvirus subfamily [1]. HSV-1 can infect a variety of cell types including neurons of the trigeminal ganglia that innervate cells at the peripheral oral-facial region.

1.1.1 *HSV-1 life cycle*

HSV-1 infects cells by attaching to cellular receptors of the cells using glycoproteins expressed on the envelope of the virus or through membrane

fusion with the cell [2]. Once inside the cell the nucleocapsid is transported to the nuclear pore, and the viral genome is transferred into the nucleus and circularizes. HSV-1 expresses three different classes of genes/proteins in a temporal fashion: immediate early (IE), early (E), and late (L) proteins [3]. The transcription of IE genes is stimulated by the tegument protein, viral protein 16 (VP16), and the cellular proteins Oct-1 and HCF. IE proteins assist HSV in evading the antiviral defenses of the host cells and transcriptionally activating E and L genes. E proteins are important for viral DNA replication, which is required to efficiently stimulate L genes. L genes are the last HSV transcripts to be transcribed and translated into proteins that are either components of virus assembly, viral structure, or viral egress from the cell.

The virus has two different life cycles: lytic and latent cycle. The lytic cycle is briefly described in the previous paragraph. After initial infection and replication of the virus, HSV-1 can then infect the neurons that innervate the oral-facial region known as the trigeminal ganglia, where the virus can become latent. When HSV-1 is latent, only the latency-associated transcripts (LATs) are actively expressed and lytic viral proteins are not translated. Various stressors, such as UV, hormone changes, fever, etc, can then reactivate the virus. This reactivation initiates active virus replication in the neurons, which eventually leads to viral replication at the primary site of infection allowing the virus to spread from person to person [1].

1.2 Immediate-early proteins

HSV-1 encodes five immediate-early (IE) proteins. The IE proteins include infected cell protein 0 (ICP0), infected cell protein 4 (ICP4), infected cell protein 22 (ICP22), infected cell protein 27 (ICP27), and infected cell protein 47 (ICP47). Both ICP4 and ICP27 are required for viral replication in cell culture [4-8]. ICP0 is an E3 ubiquitin ligase that inactivates cellular antiviral defenses and transactivates viral genes (more about ICP0 will be discussed in subsection 1.3). ICP4 is the major transcriptional activator of HSV-1 genes and is an essential viral gene required for virus replication. ICP22 is required for efficient viral replication and the formation of virus-induced chaperone-enriched (VICE) domains in cells; however, ICP22 is not essential for viral replication except in certain cell lines. The other essential IE protein, ICP27, is important for posttranscriptional processing and export of viral transcripts [9-11]. ICP47 aids the virus in evading the immune response by inhibiting transporter associated with antigen processing (TAP), so that viral antigens will not be presented on MHC Class I molecules of infected cells, which is required for the activation of cytotoxic T cells [12, 13]. Of the five IE proteins, four (ICP0, ICP4, ICP22, and ICP27) have been shown to be phosphorylated [14-16].

1.3 Infected Cell Protein 0 (ICP0)

ICP0 is a 110 kDa phosphorylated viral protein that is imperative for both efficient viral replication [17-20] and reactivation from latency [21, 22]. ICP0 has many different functions, many of which are related to its E3 ubiquitin ligase

activity. The E3 ubiquitin ligase activity is affiliated with the really interesting new gene (RING)-finger domain located within the N-terminus of ICP0 (Fig. 1.1) [23, 24]. ICP0 E3 ubiquitin ligase activity causes the degradation of many cellular proteins and disruption of nuclear domain 10 (ND10) structures [25, 26]. ICP0 is also a global gene transactivator that can transactivate all three HSV-1 gene classes [27-32]. The RING-finger domain is required for the ability of ICP0 to transactivate reporter gene expression [33], thus showing that the RING-finger domain is necessary for the transactivation activity of ICP0. The RING finger domain in ICP0 and its E3 ubiquitin ligase activity are also responsible for inactivating the cellular DNA damage response [34-36] and diminishing chromatinization and other repressive modifiers of the HSV-1 genome [37], aiding virus replication. ICP0 was also shown to inhibit interferon-stimulated genes during HSV infection [38]. Evading and/or impairing the interferon response allows HSV-1 from being inhibited by this innate response, which then allows the virus to continue to spread from cell to cell or even from person to person.

1.4 ICP0 and phosphorylation

ICP0 is a phosphoprotein and has been shown to be phosphorylated at 11 sites that are located in 3 regions of the protein (Fig. 1.1) [39], which we referred to as the Phos regions. An additional phosphorylation site was identified at site 67 (threonine) of ICP0, and this site appears to be important for the binding and degradation of the DNA damage protein, RNF8 [40]. The 3

aforementioned phosphorylated regions of ICP0 were mutated from serines (S) or threonines (T) to alanines (A) to prevent their phosphorylation, and the resulting mutations were called Phos 1, Phos 2, and Phos 3 [39]. Phos 1, with four mutated sites, has reduced transactivating activity, impaired ND10 disruption, as well as reduced viral replication in cell culture and *in vivo* (mice) [39, 41, 42]. Phos 2, which has 3 sites mutated, is impaired for ND10 disruption and showed reduced viral replication [39, 41, 42]. Phos 3, which has 4 phosphorylation sites mutated, did not have any significant phenotypes that diverged from WT HSV-1 in tissue culture or in mice [39, 41, 42].

Since the sites in region 1 seemed to have the most physiological relevance for the protein/virus, further studies were done to determine which specific sites were important for regulating specific activities of ICP0 [43]. To this end, individual sites were mutated to alanine (A) within region 1, in addition to one double mutant amino acid positions 224 and 226 [43]. It was found that S224A and double mutant S224A/T226A were more stable than WT ICP0, implying that its E3 ubiquitin ligase activity was impaired in these two mutant forms of ICP0 [43]. This increase in protein stability with S224A and S224A/T226A correlated with reduced viral replication in cell culture and *in vivo* [43].

1.5 Kinases, phosphorylation, and IE proteins

Of the five immediate early proteins, four of them (ICP0, ICP4, ICP22, and ICP27) are phosphorylated. Though the virus encodes two viral kinases, UL13

and US3, cellular kinases are also important for phosphorylating these proteins. UL13 is known to phosphorylate ICP22 [15, 44]. Phosphorylation of ICP4 appears to be important for cell replication in neurons and also during establishing latency in the mouse model [45]. ICP4 appears to be phosphorylated by protein kinase A [45, 46]. ICP27 is also a phosphoprotein, and ICP27 phos mutant viruses have impaired viral replication and impaired viral gene expression in addition to the protein losing the ability to interact with certain cellular export factors [14, 47-49]. Both UL13 and cellular kinases are shown to be important for the phosphorylation of ICP0, the latter of which will be discussed in more detail in the next section. Though we know that UL13 phosphorylates ICP0, ICP0 is also phosphorylated in the absence of other viral proteins [50], leading us to believe that cellular kinases may be important for regulation the phosphorylation state of ICP0, which in turn regulates activities of ICP0.

1.6 CDKs, the cell cycle, and ICP0

Cyclin-dependent kinases (CDKs) are an integral part of regulating the cell cycle, which is important for cell replication or proliferation. In general, CDKs phosphorylate cellular proteins that are important for cell cycle progression and transitions. These kinases are activated by being phosphorylated on a specific site when bound by specific cyclin partners. Consequently, CDKs are activated by distinct cyclins depending on which specific phase of the cell cycle one or more cyclins are expressed; in contrast

CDKs tend to be expressed at fairly constant or steady state levels throughout the cell cycle. Given that many CDKs are active during different times in the cell cycle, these kinases have the capability of phosphorylating different targets. Of the CDKs associated with the cell cycle, CDK4 and CDK6 are important for progression in the G₁ phase [51, 52] and phosphorylate certain cellular proteins such as retinoblastoma tumor suppressor protein at the G₁ to S phase transition [51, 52]. CDK1 controls G₂ phase, the G₂/M transition, and M phase of the cell cycle. CDK2 is active during the G₁/S transition [51, 52] and S [53] phases of cellular proliferation.

Although ICP0 is known to be highly phosphorylated, as previously discussed in section 1.4, not much is known about which specific cellular kinases influence its phosphorylation state. Published studies have been performed with the CDK inhibitor, Roscovitine (Rosco), which inhibits CDK1, CDK2, likely CDK3, CDK5, CDK7, and CDK9. Rosco impaired the transactivation activity of ICP0 [54], an important function of ICP0, establishing a connection between ICP0 and CDKs. In addition to its impaired transactivation activity, the posttranslational modification status of ICP0 is altered by Rosco, suggesting the Rosco-sensitive CDKs may phosphorylate ICP0 [54]. ND10 disruption (e.g., dissociation of PML and Sp100) was not, however, affected in HSV-1-infected cells treated with Rosco [55]. Rosco also impaired HSV-1 replication in cell culture and reactivation by explanting latently infected tissue [56, 57]. During the course of explant-induced reactivation in HSV-1 infected mouse trigeminal ganglia, CDK2 and/or CDK4 expression increased over time

[57], potentially linking CDK expression to ICP0, as ICP0 is required for efficient reactivation (Halford *et al.*, 2001, Halford and Schaffer, 2001). Expression of ICP0 during viral infection or in the absence of over viral proteins has been shown to block cellular proliferation at the G₁/S and G₂/M transitions [58, 59]. Interestingly, these transition phases require CDK1, CDK2, CDK4 and CDK6. This does put forth the possibility that ICP0 and CDKs regulate one another. Another potential relationship between ICP0 and specific CDKs come from bioinformatics studies regarding regions of ICP0 phosphorylation; these studies indicate that regions I and II of ICP0 phosphorylation contain potential CDK1 and CDK2 consensus phosphorylation sites [39]. Furthermore, CDK1 has been shown to phosphorylate exon 1 of ICP0 *in vitro* [60], and a purified GST-CDK4 fusion protein has been shown to interact with ICP0 in pull-down from infected cell extracts [61].

Though we know that Rosco (CDK inhibitor) inhibits ICP0 transactivation [54], that specific CDKs can phosphorylate and/or interact with ICP0 [60, 61], and that phosphorylation regulates activities of ICP0 [39-43, 54, 55], we don't know which specific cellular kinases are responsible for regulating the activities of ICP0. In order to begin to elucidate which specific cellular kinases are important for regulating activities of ICP0, we decided to initially develop a high throughput cell-based assay with an siRNA library to identify which potential cellular kinases are important for the transactivation activity of ICP0 described in Chapter 2 [62]. After identifying a number of cellular kinases that appeared to be important for ICP0's transactivation activity, we decided to focus on CDK1,

CDK2, CDK4, and CDK6 due to the connection with ICP0 and these specific cellular kinases in previously published reports [54, 58-61] and from the results in our siRNA screen (Fowler, *et. al.*, in prep). We took the approach to determine which specific CDKs are important for regulating the activities of ICP0 and HSV-1 by using specific CDK inhibitors. We determined at that the activities of two CDKs are crucial for controlling ICP0 activities and HSV-1 replication and reactivation, as will be discussed in Chapter 3.

1.7 Figure and Figure legends

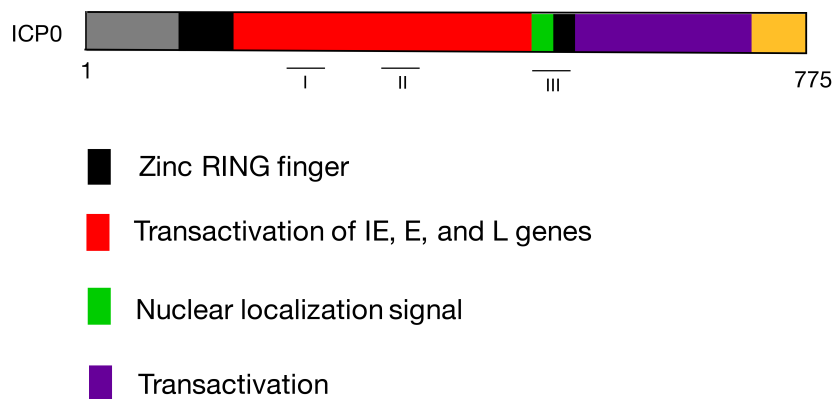


Figure 1.1 HSV-1 ICP0 structure and functional domains.

This figure has been modified from Davido, *et. al.*, 2005. Select functional domains of ICP0 are indicated. Phosphorylation regions are labeled in I, II, and III.

Chapter 2: Development of a novel cell-based assay to monitor the transactivation activity of the HSV-1 protein ICP0

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2.1 Introduction

Herpes simplex virus type 1 (HSV-1) infects 70-80% of the population and commonly causes cold sores, although many infections are asymptomatic [1]. HSV-1 can also cause ocular infections and is the major cause of infectious blindness in western industrialized countries [1]. Initially, HSV-1 infects epithelial cells at a primary site, resulting in a lytic infection. The virus can then infect the sensory neurons that innervate the infected epithelial cells, where it establishes a life-long latent infection. Reactivation of latent virus can occur by different stresses, which initiates lytic infection in neurons and ultimately epithelial cells at the primary site of infection. This cycle of latent and lytic infections leads to recurrent disease. One viral protein that is important for efficient for viral replication and reactivation from latency is the immediate-early (IE) protein, infected cell protein 0 (ICP0) [17, 27, 63].

ICP0 is one of the five HSV-1 IE proteins and has RING-finger motif [64, 65] that allows ICP0 to function as an E3 ubiquitin ligase [66]. E3 ubiquitin ligases are components of an E1-E2-E3 biochemical pathway that conjugates and polymerizes chains of ubiquitin onto proteins [67]. This activity of ICP0 directs the degradation of specific cellular proteins [25, 26, 68-71], which in turn impairs cellular antiviral defenses. The E3 ubiquitin ligase activity of ICP0 is also linked to the ability of ICP0 to act as a potent global transcriptional activator of viral genes [72, 73]. Previous studies have shown that the transactivation activity of ICP0 is important for efficient HSV-1 replication and reactivation from latency [18, 74, 75]. Although the transactivating activity of ICP0 has been studied in the absence of other IE genes in transient transfection assays [33, 39, 43, 76], it has not been extensively studied independent of other IE proteins in the context of a viral infection. A limited number of cellular factors have been linked to ICP0's transactivation activity [54, 77]; however, the development of a cell-based assay to monitor this activity would allow for the systematic identification of key cellular factors or activities (e.g., via siRNA knockdown) that contribute to this function.

To begin to elucidate cellular factors that affect ICP0's transactivating activity, we have developed an assay that measures this activity of ICP0 in a 96-well plate format. The assay was developed using two HSV-1 reporter viruses that express only ICP0 of the 5 IE genes (*d106*) or none of the IE genes (*d109*). Both viruses have a human cytomegalovirus major immediate early promoter (HCMV MIEp) driven green fluorescent protein (GFP) in the HSV-1 *ICP27* locus

[78]. Similar to other published reports [38, 75, 79, 80], we show here that ICP0 is capable of transactivating the HCMV MIEp; in the case of *d106*, ICP0 transactivation activity is quantified by measuring GFP fluorescence levels using a Typhoon Imager. To ultimately adapt this screen to use siRNAs against cell targets, we demonstrate that ICP0's transactivating activity was minimally affected when cells were transfected with an siRNA against the cellular house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Using a 96-well plate format, we established that *d106* (+ICP0) has higher levels of GFP than *d109* (-ICP0), which increase from 12 to 24 h post infection (hpi). Lastly, we use this assay in an siRNA library screen and identify that an siRNA against ubiquitin activating enzyme E1-like (*UBEL1*) impairs the transactivation activity of ICP0, validating the development of our assay.

2.2 Materials and Methods

2.2.1 *Cell culture and viruses*

HeLa S3 (human cervical carcinoma) cells (kindly provided by Ira Blader) were utilized in the assay and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 U/mL penicillin, 10 U/mL streptomycin, and 50 µg/mL gentamycin at a temperature of 37°C in 5% CO₂.

HSV-1 recombinant viruses, *d106* and *d109* (Fig 2.1, modified [78], kindly provided by Neal DeLuca) were used in our cell-based assay. Of the 5 IE genes, *d106* only expresses ICP0 (+ICP0), whereas *d109* does not express any IE

proteins (-ICP0). Both viruses express the GFP gene from the human cytomegalovirus major IE promoter (HCMV MIEp). Both *d106* and *d109* viruses were grown on M17 cells, which are a derivative of Vero cells stably transformed with the HSV-1 ICP4 and ICP27 genes (kindly provided by David Leib). Adenoviral vectors, Ad-crtTA and Ad-T-ICP0, were used to infect M17 and transiently express ICP0 to increase titers of *d109*. Ad-crtTA and Ad-TICP0 were grown and titered as previously described [22]. *d106* was titered on Vero cells and *d109* was titered on L7 cells (Vero cells that contain the ICP0 gene) [81], and fluorescent forming units were counted 24 hpi. M17, Vero, and L7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 10 U/mL penicillin, 10 U/mL streptomycin, and 50 µg/mL gentamycin at a temperature of 37°C in 5% CO₂.

2.2.2 Reverse transfection

HeLa S3 cells were reverse transfected in BD Falcon black well clear bottom optilux 96-well plates (San Jose, California, catalog # 08-772-104). Ambion siRNAs (Grand Island, NY) were used at a volume of 10 µL per well and final concentration of 10 µM per well. The ubiquitin pathway siRNA library (Ambion, Grand Island, NY, Catalog number #AM80991V3) was used at the same volume and concentration. siRNA was pre-plated in wells of the 96-well plate. 0.2 µL of Lipofectamine 2000 per well was mixed with 9.8 µL Opti-MEM per well for 10-25 min. The Lipofectamine 2000/Opti-MEM mix is added to the pre-plated siRNA and allowed to incubate for 20 minutes. After the 20 min

incubation, HeLa S3 cells were added to the wells at 8,000 cells per well in 80 μ L per well in penicillin/streptomycin free 10% FBS DMEM. Transfected and mock-transfected wells were washed twice with complete medium 24 h post transfection (hpt) and then 100 μ L of HeLa S3 cell medium was added to each well. 48 hpt, medium was removed from the cells and the well was either infected with previously described reporter viruses or harvested for RNA isolation.

2.2.3 Infection

HeLa S3 cells were infected 48 hpt with *d*106 or *d*109 reporter viruses at a multiplicity of infection (MOI) of 1. Viruses were diluted in HeLa S3 medium that did not contain phenol red. Infections were allowed to proceed for 12, 18, and 24 hpi.

2.2.4 Detection and analysis of fluorescent signals

Plates were scanned using a Typhoon image scanner (General Electric Healthcare) at standard settings according the manufacturer's recommendations. Settings were chosen to scan green fluorescence at an excitation of 488, emission of 520, and photomultiplier tube (PMT) of 475 and excitation of 633, emission of 670, and photomultiplier tube (PMT) of 475 for the deep red. Scans were done at 12, 18, and 24 hpi. At the 24 h time point for the siRNA screen, cells were stained with deep red mitotracker in FBS-free DMEM at a final concentration of 250 nM as per protocol (Invitrogen, Grand Island, NY, catalog # M22426) to account for cell density. The fluorescence intensity of

each scanned well was analyzed in ImageJ by using a circle the size of the well & measuring the intensity of each well. Data was copied into Excel (Microsoft) software for analysis. Wells that contained only cells and medium were used as background controls, and this value was subtracted from values of infected wells. For the siRNA screen, the GFP intensity was normalized to the mitotracker (deep red) intensity. *d106* values were set at 100% and mock transfected, *GAPDH* siRNA transfected *d106*, *d109* values were compared to the 100% value. For the siRNA screen, similar calculations were done, but normalized values were used instead of GFP only values. Lastly, the percent average variation for all *d106* signals using the data in presented in Fig. 2.5 was calculated to be 3% of the mean *d106* signal.

2.2.5 RNA isolation and reverse transcription real time PCR

Cells were lysed and harvested using Trizol (Invitrogen, Grand Island, NY) according to manufacturer's recommendations. RNA was isolated using phenol-chloroform extraction. 500 ng of RNA was reverse transcribed to make cDNA using the iScript BioRad cDNA synthesis kit (BioRad, Hercules, CA, catalog # 170-8890) according to manufacturer's conditions. Real time PCR was done by using SYBR green master mix (Roche) and standard conditions were used for the cellular target, *GAPDH* (5'-CGGATTTGGTCGTATTGGGCGC-3' and 5'-TCCCGTTCTCAGCCTTGACGGT-3'), and the normalization control, *hTBP* (5'-TGCACAGGAGCCAAGAGTGAA-3' and 5'-CACATCACAGCTCCCCACCA-3') [82]. Standard curves were obtained for the

target and normalization control. Comparisons were made using the $\Delta\Delta^2CT$ method, setting the untransfected control at 100%.

2.3 Results

2.3.1 *ICP0 transactivates the HCMV MIEp-GFP.*

With the long-term goal of identifying cellular factors that play a role in the transactivating activity of ICP0, we sought to develop a 96-well plate cell-based assay. We initially wanted to establish if the replication-defective reporter viruses, *d106* (+ICP0) and *d109* (-ICP0) [78], could be used in this format. Specifically, we wanted to use fluorescence as an output of ICP0's transactivation activity. Both *d106* and *d109* viruses contain an HCMV MIEp-GFP reporter, and previous studies indicate that ICP0 can transactivate the HCMV MIEp (Fig. 2.1). We first tested whether the expression of GFP is dependent on ICP0 in HeLa cells, a cell type commonly used to study HSV-1 replication that is highly transfectable for nucleic acids (e.g., siRNA and DNA plasmids) [33, 83]. HeLa S3 cells were infected with *d106* (+ICP0) or *d109* (-ICP0), and GFP expression was examined by microscopy at 2, 6, 12, and 24 h post infection (hpi). Activation of the HCMV MIEp-GFP was first detected by 6 hpi for *d106* (+ICP0) and steadily increased until 24 hpi (Fig. 2.2). In contrast, activation of HCMV MIEp-GFP in *d109* (-ICP0) became apparent by 12 hpi in a subset of cells (Fig. 2.2), with increased GFP expression by 24 hpi (Fig. 2.2). Although the GFP is induced in *d109* (-ICP0)-infected cells, it does not reach the level, both in relative fluorescence intensity and cell number, as *d106* (+ICP0)-

infected cells. This experiment indicated that ICP0 potentially induced the HCMV MIEp-GFP reporter construct in *d106*; thus, we established that *d106* can be used as a reporter virus for monitoring ICP0 transactivating activity.

2.3.2 siRNA depletion of GAPDH does not appear to affect ICP0's transactivation activity by fluorescence microscopy.

We next wanted to know if transfecting an siRNA against a cellular housekeeping gene would affect the transactivating activity of ICP0. For this experiment, HeLa S3 cells were untransfected, mock transfected, or transfected with an siRNA targeted against *GAPDH*. Mock-transfected cells were included in these experiments to determine if the transfection reagent alone modulated ICP0's transactivating activity. As indicated in Figure 2.3, we observed no notable differences in GFP levels of *d106* (+ICP0)- or *d109* (-ICP0)-infected cells that were untransfected, mock transfected, and *GAPDH* siRNA-transfected via microscopy.

2.3.3 GAPDH transcript levels are depleted in a 96-well plate format.

Once we showed that transfection of siRNAs into cells did not appear to affect GFP levels of *d106* (+ICP0) or *d109* (-ICP0)-infected cells as imaged by microscopy, we next wanted to confirm that we could deplete the transcript levels of a cellular target using an siRNA in a 96-well plate format. HeLa S3 cells were untransfected, mock transfected, or transfected with a siRNA targeting *GAPDH* in triplicate wells. Forty-eight h post transfection (hpt), RNA from each well was isolated, reverse transcribed into cDNA, and analyzed by Real Time

PCR. Transcript levels of *GAPDH* were significantly reduced to 3.4% of untransfected levels in the siRNA-transfected samples and to 75% in mock-transfected samples compared to untransfected samples, which were given the value of 100% (Fig. 2.4). This experiment shows that we are able to reduce mRNA levels of a specific cellular target in a 96-well plate substantially.

2.3.4 *An siRNA against GAPDH has a minimal affect on ICP0's transactivation activity in a 96-well plate.*

After we established that an siRNA against *GAPDH* depleted its transcript levels using a 96-well plate, we adapted this assay using the *d106* and *d109* reporter viruses to a 96-well plate format. HeLa S3 cells were untransfected, mock transfected, or transfected with a siRNA against *GAPDH*. At 48 hpt, HeLa S3 cells were mock infected in triplicate or infected with *d106* or *d109* at an MOI of 1. The 96-well plate was scanned for fluorescence using a Typhoon Imager at 12 hpi, 18 hpi, and 24 hpi. GFP expression was detected at 12 hpi for *d106* (+ICP0) (Fig. 2.5A), which gradually increased at 18 and 24 hpi (Fig. 2.5A). For each time point, *d106* (+ICP0) GFP levels are visibly higher than *d109* (-ICP0), with *d109* GFP intensities were reduced 5-6 fold at 12 hpi, 9.5-12 fold at 18 hpi, and 10.4-14 fold at 24 hpi (Fig. 2.5B) relative to *d106*, whether in the absence or presence of the *GADPH* siRNA. Lastly, there results confirmed our microscopy studies in which an siRNA against *GAPDH* did not appreciably affect GFP intensities for *d106*- or *d109*-infected wells compared their respective untransfected controls (Fig. 2.5B). These experiments demonstrate that we can

examine the transactivation activity of ICP0 using *d106* in a cell-based assay in a 96-well plate format.

2.3.5 *An siRNA against UBE1L impairs the transactivation activity of ICP0.*

Utilizing our 96-well plate cell-based assay, we screened a small siRNA library that targeted components of the ubiquitin pathway. Wells were reverse-transfected and infected with *d106*. From this screen, an siRNA against *UBE1L*, an E1 enzyme of the ubiquitin-proteasome pathway, impaired the transactivation activity of ICP0 5.5-fold at 24 hpi (Fig. 2.6), which began to approach *d109* (-ICP0) GFP levels (Fig. 2.6). Thus, we demonstrate that this assay can be used in an siRNA screen having identified a target in the ubiquitin-proteasome pathway.

2.4 Discussion

In this paper, we have demonstrated, in line with other studies [75, 79, 80], that ICP0 can potently transactivate the HCMV MIEp (Fig. 2.2). We also found that an siRNA against the housekeeping gene, *GAPDH*, does not appear to affect the transactivation activity of ICP0 as measured by microscopy (Fig. 2.3) and that *GAPDH* transcripts could be depleted to significant levels in a 96-well plate format (Fig. 2.4). These results indicate that siRNA technology can be used in this cell-based assay in a 96-well plate format. We also established that the Typhoon Imager is a quick and valid method to examine ICP0's transactivating activity in a 96-well plate format (Fig. 2.5), and we verified that

the assay can be used to screen an siRNA library by identifying an ubiquitin-proteasome pathway target, UBE1L (Fig. 2.6). Overall, we have concluded that the reporter viruses, *d106* and *d109*, can be utilized in a cell-based assay by using a 96-well plate format and Typhoon image scanner.

While this system is adaptable to siRNA or small molecule inhibitor screens, this method also has several advantages over other established methods used to examine ICP0's transactivation activity [33, 43, 54, 76]. Specifically, ICP0's transactivation activity can be monitored in the context of a viral infection, in the absence of other IE proteins, and at multiple time points in one experiment or infection by the assaying of live cells. In contrast, other methods monitor this activity in the context of a viral infection but in the presence of other IE proteins [54], which may complicate the interpretation of certain studies. Transient transfection reporter assays have been used to examine ICP0's transactivation activity independent of other IE proteins, but these assays are not done in the setting of a viral infection [33, 43, 76]. Finally, most viral reporter assays and transient transfection assays can only monitor one time point per sample because the cells will either have to be lysed or fixed to analyze the sample, in contrast to our system. Thus, the *d106/d109* reporter assay is advantageous because it can be used to monitor multiple time points for one experiment and monitor ICP0's transactivation activity in the absence of IE proteins while in the context of a viral infection. Furthermore, the inclusion of *d109* can also be used in counter-screens to verify that targets or inhibitors are specific for ICP0.

In summary, we have developed a cell-based assay for ICP0 that can use siRNA technology or small molecule inhibitors in a 96-well plate format. This assay can also be modified for use in a high throughput screen with the inclusion of viability indicators such as alamarBlue [84]. Notably, this assay will likely increase our knowledge related to the regulation of ICP0's transactivating activity, which may eventually lead to potential treatments against HSV-1 and its recurrent diseases.

Contribution to this work: I did all the experimental design, setup, and analyses described in this chapter, with the exception for the set up of data analyses.

2.5 Figures and Figure Legends

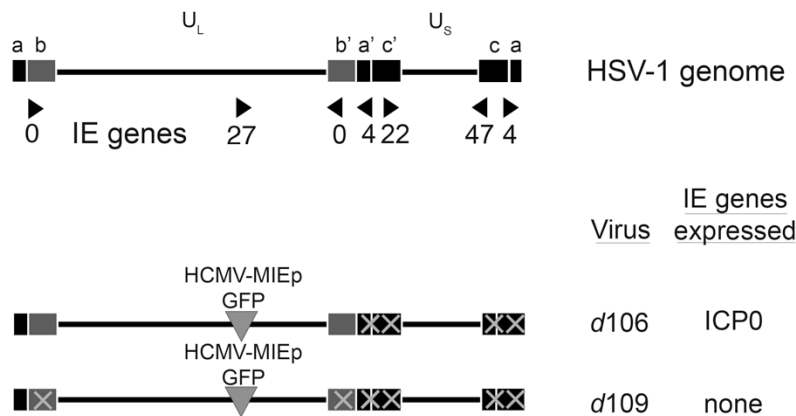


Figure 2.1 HSV-1 recombinant viruses, *d106* and *d109*, utilized in our cell-based assay.

Of the 5 IE genes, *d106* only expresses ICP0 (ICP0+), whereas *d109* does not express any IE proteins (ICP0-). Both viruses express the *GFP* gene from

the human cytomegalovirus major IE promoter (HCMV MIEp). This figure is modified from Samaniego, *et. al.*, 1998.

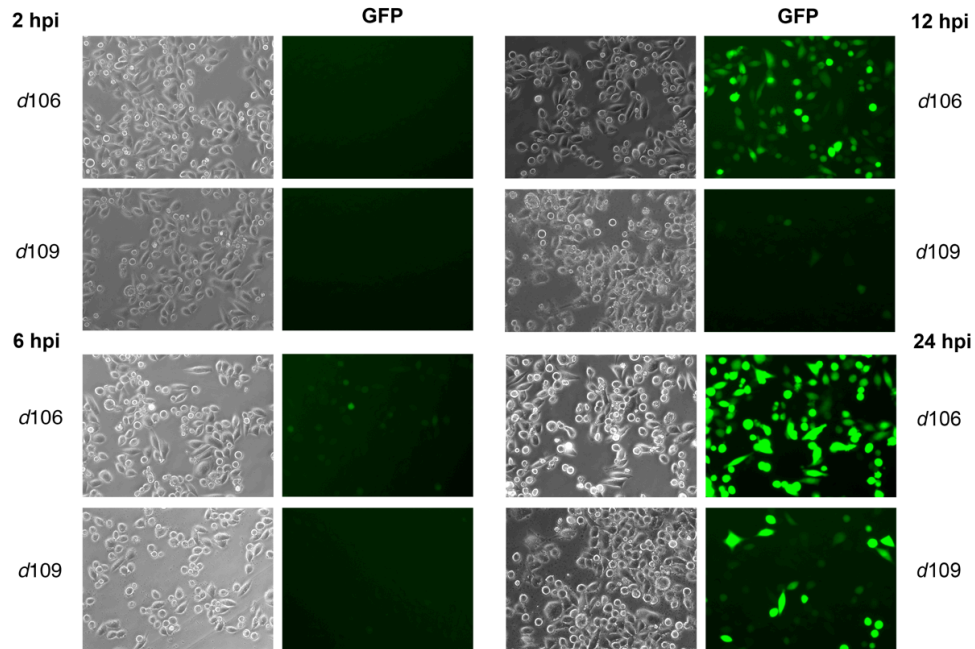


Figure 2.2 ICP0 transactivates the HCMV MIEp-GFP.

HeLa S3 cells were seeded in a 12-well plate, and mock-infected or infected at an MOI of 1 FFU per cell of *d106* or *d109* per cell. Cells were examined by fluorescence microscopy at 2, 6, 18, and 24 h post infection (hpi), and images were captured with a digital camera. Light microscopy images are located on the left and fluorescent images are located on the right for each virus.

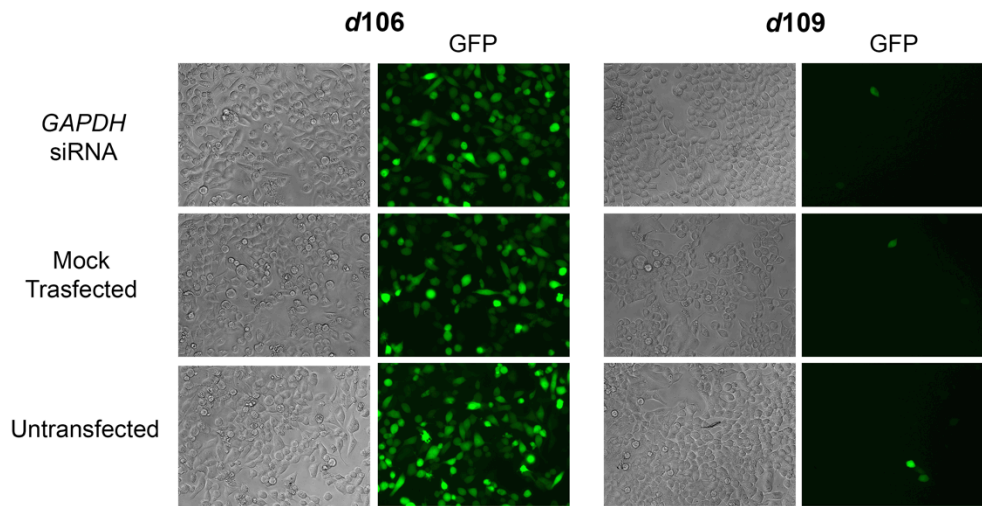


Figure 2.3 siRNA depletion of *GAPDH* does not affect ICP0's transactivation activity.

HeLa S3 cells were untransfected, mock transfected or transfected with an siRNA directed against *GAPDH*. Mock-transfected cells were included in these experiments to determine if the transfection reagent alone modulates ICP0's transactivating activity. All samples were compared to the untransfected control. Forty-eight hpt, HeLa S3 cells were infected with an MOI of 1 with d106 or d109 for 24 h and viewed by fluorescence microscopy. Light microscopy images are located on the left and fluorescent images are located on the right per virus. Images were captured with a digital camera.

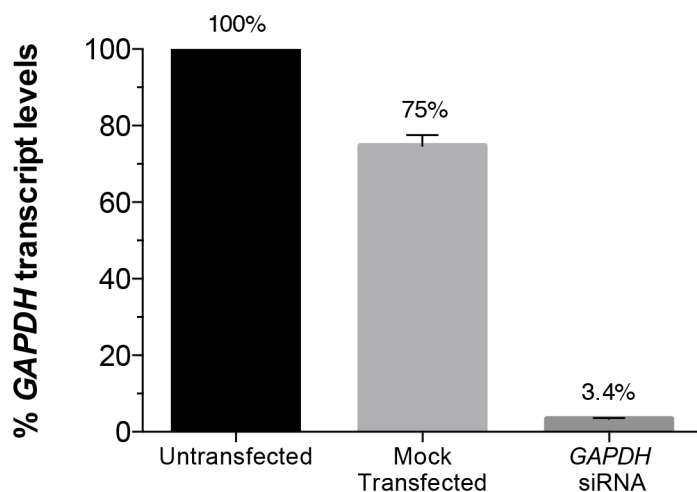


Figure 2.4 *GAPDH* transcript levels are depleted in a 96-well plate.

HeLa S3 cells were untransfected, mock transfected, or transfected with a siRNA targeting *GAPDH* in triplicate wells in a 96-well plate. Twenty-four hpt, well were washed as described in materials and methods. Forty-eight hpt, RNA was isolated from each well, reverse transcribed into cDNAs, and analyzed by Real Time PCR. Comparisons were made using the $\Delta\Delta^2$ CT method. *GAPDH* transcript levels were compared between mock- and *GAPDH*-transfected cells relative to the untransfected control, which was set at 100%. Error bars represent SEMs. This experiment was repeated in triplicate 3 times.

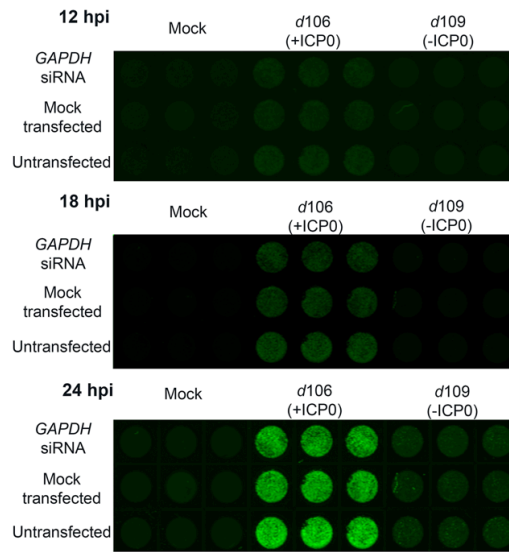
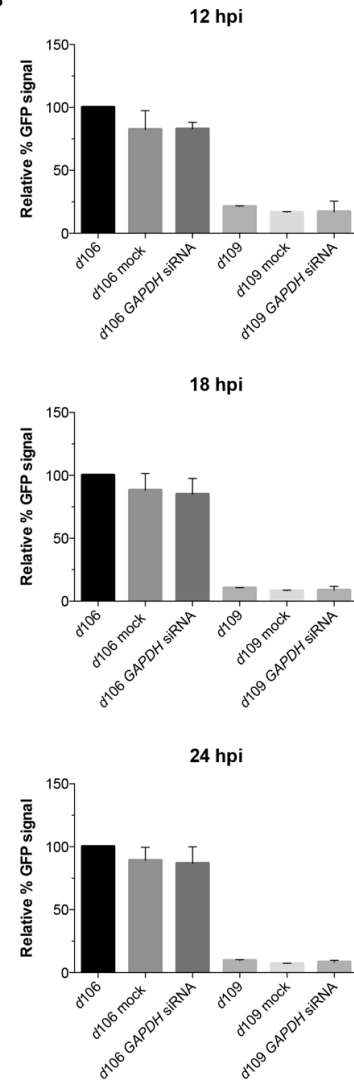
A**B**

Figure 2.5 An siRNA against *GAPDH* has a minimal effect on ICP0's transactivation activity in a 96-well plate format.

HeLa S3 cells were untransfected, mock transfected, or transfected with an siRNA against *GAPDH*. At 48 hpt, HeLa S3 cells were mock infected or infected with *d106* or *d109* at an MOI of 1. (A) The 96-well plate was scanned using a Typhoon Imager at 12 hpi, 18 hpi, and 24 hpi. This experiment was repeated in triplicate 2 times. (B) Comparisons of *d106* and *d109* samples –

untransfected *d*106 samples were set at 100% for all time points, and mock transfected and siRNA-transfected samples were compared to it. Error bars represent SEMs.

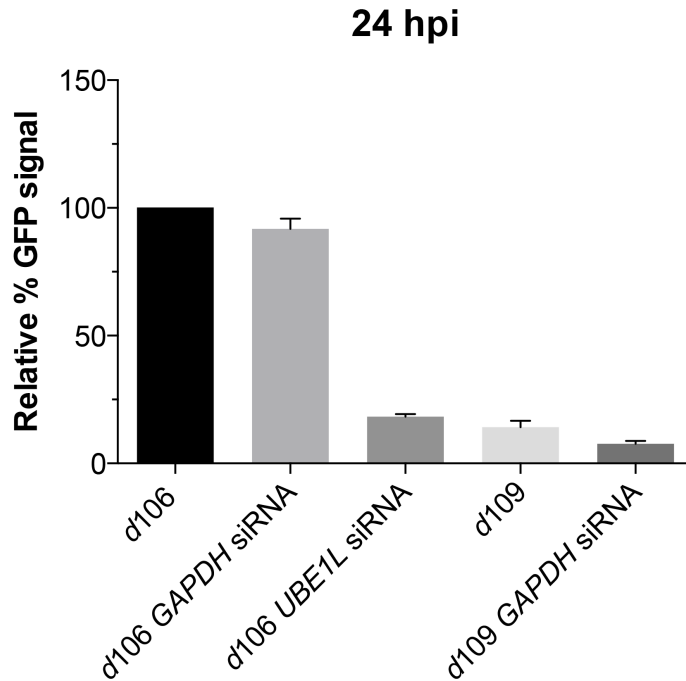


Figure 2.6 An siRNA against *UBE1L* impairs the transactivation activity of ICP0.

HeLa S3 cells were seeded in a 96-well plate. Cells were reverse transfected as described in the protocol. Wells that contained siRNA against the ubiquitin ligase pathway were infected with *d*106 at an MOI of 1, 48 hpt. Cells were untransfected or transfected with *GAPDH* siRNA for controls. 48 hpt, control wells were infected *d*106 or *d*109 or mock infected. The 96-well plate was scanned using a Typhoon Imager at 24 hpi. Comparisons were made relative to *d*106-untransfected infection, which was set at 100% (far left bar for

each graph). *UBEL1* is ubiquitin-activating enzyme E1-like. Error bars represent SEMs. This experiment was repeated 3 times.

Chapter 3 Activities of one or more CDKs play roles in HSV-1 gene expression and replication and facilitate reactivation from a quiescent infection in an ICP0-dependent manner.

3.1 Introduction

Herpes simplex virus type 1 (HSV-1) infects 70%-80% of the world population [1]. Herpes simplex virus type 1 (HSV-1) typically infects fibroblasts and epithelial cells on or around the mouth and lips. Primary infection caused by HSV-1 is typically mild or asymptomatic; however, HSV-1 can also cause very severe diseases such as infectious blindness and encephalitis. The virus can exist in two states of infection: lytic and latent. The lytic infection of HSV-1 is characterized by a temporal cascade of gene expression including three classes of genes: immediate early (IE), early (E), and late (L) [85]. Once HSV-1 replicates in the initial site of infection, it then infects neurons that innervate the primary infection site. It can establish a latent state in neurons that is characterized by limited viral gene expression and a lack of infectious virus [86]. Various stressors can cause the virus to reactivate, leading to lytic infection in neurons and cells at the initial site of infection. The IE viral protein infected cell protein 0 (ICP0) plays an important role in both efficient lytic infection and reactivation from latency [17, 18, 63].

ICP0 is one of five IE proteins expressed by HSV-1 and is a multifunctional phosphorylated protein that has a zinc RING-finger motif that controls its E3 ubiquitin ligase activity [66]. ICP0 transactivates all three classes

of HSV-1 genes as well as non-HSV-1 genes [27, 30-32, 87]. As an E3 ub ligase, it has ability to degrade components of the host's antiviral nuclear domain (ND) 10 [25, 26] and interferes with the chromatinization of viral DNA [88-91]. All of these activities of ICP0, including its ability transactivate viral genes, are linked to its E3 ub ligase activity [25, 72].

It has been known for several decades that ICP0 is a phosphorylated protein [14], and this post-translational modification appears to regulate many of its functions [39-43]. Phosphorylation sites in each of three identified regions (termed 1, 2, and 3) were blocked by site-directed mutagenesis, creating the mutant forms of ICP0: Phos 1, 2, and 3 [39]. Out of the three Phos mutants generated, Phos 1 showed the greatest impairment for transactivation activity [39, 41], E3 ub ligase activity [41], increased ICP0 stability [41], and reduced replication in cell culture and in mice [41] and reduced reactivation [42]. Within this region, the serine at 224 was largely responsible for these phenotypes [43]. Phos 2 was impaired for replication in the eyes and trigeminal ganglia of mice and for reactivation from latency by explanation [42]. Mutation of region 3 appeared to replicate and reactivate similarly to WT HSV-1.

Although phosphorylation has been linked to the regulation of ICP0 activities, little is known about the specific kinases that are involved in this regulation. While HSV-1 encodes two viral kinases, US3 and UL13, ICP0 is extensively phosphorylated in cell culture in the absence of other viral proteins [50]. This observation suggests that cellular kinases are important for the phosphorylation and regulation of ICP0. CK1 has been shown to phosphorylate

T67 in ICP0, which is important for recruiting and subsequently degrading RNF8 [40]. Studies with the cyclin-dependent kinase (CDK) inhibitor, Roscovitine (Rosco), which inhibits CDK1, CDK2, CDK5, CDK7, and CDK9, have linked the activities of CDKs to the functions of ICP0 [39]. Specifically, Rosco impaired ICP0's transactivation activity and altered the posttranslational modification state of ICP0 [54]. In another study, ICP0 was phosphorylated by CDK1 *in vitro* [60]; however, the biological consequence of this phosphorylation event was not examined. Additionally, an interaction between ICP0 and CDK4 was identified via co-immunoprecipitation assays using bacterially expressed CDK4 [61], and it was observed that CDK6 is recruited to the nucleus of cells in the presence of a CDK4 inhibitor during infection [61]. Furthermore, bioinformatics programs identified CDK1 and/or CDK2 to be potential kinases that can phosphorylate residues in region 1 and 2 of ICP0 phosphorylation [39].

Since little is known about which specific cellular kinases are necessary for regulating the activities of ICP0, we sought to examine this area of research. For this investigation, we performed a high throughput screen (HTS) using an established cell-based assay [62] and an siRNA library that targeted (known and putative) cellular kinases to determine which of these kinases may play a role in ICP0's transactivation activity. While we identified a number of cellular kinases from our HTS siRNA screen as potential regulators of ICP0 transactivation activity, we focused our efforts on CDK1, CDK2, CDK4, and, CDK6 because of the results from our screen and data from previously published reports that link these CDKs to ICP0 [54, 60, 61]. For our functional studies we used CDK

inhibitors of CDK1, CDK2, and CDK4/6 to study different activities of ICP0 in the absence of specific CDK activities. We found that specific CDK inhibitors do not affect PML and sp100 dispersion mediated by ICP0. Inhibitors of CDK1 and CDK2 (termed CDK1i and CDK2i, respectively) impaired WT HSV-1 replication; however, the CDK2i impaired the replication of an ICP0-null virus, implying that the CDK1 activity is required for ICP0-specific replication. CDK1i reduces UL42 protein expression while CDK2i diminished the levels of several E and L proteins during WT HSV-1 infection. CDK1i and CDK2i also reduced HSV-1 reactivation from a model of quiescent infection. Based on our results, we conclude that CDK1 appears to regulate the expression of one HSV-1 protein, viral replication, and reactivation from a quiescent infection in an ICP0-dependent manner, whereas CDK2 appears to regulate viral replication in ICP0-independent manner and reactivation in an ICP0-dependent manner.

3.2 Materials and Methods

3.2.1 *Cells and Viruses*

Vero cells were obtained from American Type Culture collection (ATCC) and cultured in Dulbecco's Modified Eagle's medium (DMEM) that was supplemented with 5% fetal bovine serum (FBS), 100 µg/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine as described previously [81]. HeLa S3 cells were generously provided by Dr. Ira J. Blader and cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 µg/mL penicillin, and 100 µg/mL streptomycin. HepaRG cells were generously provided by Drs. Roger

Everett and Chris Boutell and are a liver cell line used to study HSV and ICP0. HepaRG cells were cultured in William's E medium supplemented with 10% FBS, 2 mM glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, and 500 nM hydrocortisone. L7 cells, a Vero cell line that stably expresses HSV-1 ICP0 is described in [81], were cultured in the same manner as Vero cells. HFT cells are an immortalized Human Foreskin Fibroblast (HF) cell line and were generously provided by Chris Boutell and described and maintained as previously described [92]. *d106* and *d109* (Fig. 3.1) were used for in a high throughput screen and are grown as previously described [62]. *d106* expresses only ICP0 of the five IE genes, while *d109* doesn't express any IE genes. Both *d106* and *d109* have a HCMV IE promoter-GFP cassette inserted into the *UL54* locus. WT KOS and 7134 (ICP0-null mutant derived from KOS strain) were propagated as previously described [18, 27]. Titers of KOS were determined via plaque assays on Vero cells, while 7134 titers were performed on L7 complementing cells. *in1374* was used as an indicator virus in our HSV reactivation studies and was generated and propagated as described in [93]. *d/x3.1*, an ICP0-null mutant used as a negative control in our reactivation studies, was propagated as described in [94] and titered on L7 monolayers.

3.2.2 High throughput siRNA cell based assay

A high throughput cell-based assay was adapted for our siRNA screen as described in publication [62]. Ambion siRNA against known and putative cellular kinases (catalog #AM80991V3) was used in the assay. Transfected-Infected

plates were scanned by a Typhoon fluorescence imager (GE Healthcare) at 12, 18, and 24 hpi to monitor GFP fluorescence. Mitotracker fluorescent dye was used [250 nM] to determine relative cell density of each well at 24 hpi. The relative fluorescence signal of each well was determined with the software program, ImageJ, as previously described[62].

3.2.3 *CDK Inhibitors and cell cycle profile*

Specific CDK inhibitors: CDK1 inhibitor CGP74514A (CDK1i), CDK2 inhibitor III (CDK2i), and CDK 4/6 inhibitor IV (CDK4/6i), were purchased from EMD Millipore Corporation. To determine the appropriate concentration of each inhibitor that blocked cell cycle progression, various inhibitor concentrations were added to HepaRG cells (5×10^5) in 60 mm dishes, and cells were fixed with ethanol and treated with RNase. Cellular DNA was stained using propidium iodide (PI), and DNA content was analyzed by Accuri C6 flow cytometer and Flowjo software. The viability of the CDKi treated cells was calculated by staining cells with a 1:1 (vol:vol) ratio of 0.4% Trypan blue and live and dead cells were counted. Percent cell viability were determined based on live number of cells over the total number of cells. From these experiments, CDK1i was used at a concentration of 2.5 μ M, CDK2i was used at a concentration of 17 μ M, and the CDK4/6i was used at a concentration of 2 μ M. The broad CDK inhibitor, Rosco which inhibits CDK1, 2, likely 3, 5, 7, and 9, was used as a positive control at a final concentration of 75 μ M. Drugs were prepared in DMSO as

described per manufacturer. Inhibitor studies were performed using a modified cycloheximide (CHX) block and release protocol as discussed [54].

3.2.4 Immunofluorescence assays

HepaRG cells were seeded at 5×10^4 cells per well on collagen-coated coverslips in a 24-well plate. Twenty-four hours post seeding, cells were treated with CHX. One hour post CHX-treatment, cells were infected with KOS at an MOI of 2. Four hours post infection, cells were washed with phosphate buffered saline (PBS) and release from the CHX block. Specific CDK inhibitors or Rosco was added 3.5 hpi and again after release of the CHX block. At 2 hours post release (hpr), cells were fixed, permeabilized, and washed as described in Mostafa, *et. al.*, 2013, except a concentration of 3.7% formaldehyde was used instead of 5% to fix the cells. Cells were blocked for 1 hour at 37°C with PBS-FB containing 1% FBS and 1% bovine serum albumin (BSA).

3.2.4.1 PML and ICP0 staining

Cells were stained with primary and secondary antibodies, mounted, and examined as described in [43]. Cells were viewed using a Nikon fluorescent microscope and imaged using a CoolSNAP EZ digital camera and NSI Elements software. Images were taken at the same exposure time and processed using Adobe Photoshop software.

3.2.4.2 sp100 and ICP0 staining

Infected HepaRG cells were stained with primary and secondary antibodies as described in [71]. Again, cells were viewed with a Nikon

fluorescent microscope and imaged with a CoolSNAP EZ digital camera and NSI Elements software.

3.2.5 *Viral Replication Assays*

HepaRG cells were seeded in 12-well plates at a concentration of 1×10^5 cells per well. 24 h post-seeding, a CHX block and release was performed as described as above. One hour after CHX was added, wells were infected with KOS or 7134 with a MOI of 1 PFU/cell. Infections were synchronized by washing the wells with PBS three times 1 hpi, then media with CHX was added to the cells. 3.5 hpi, samples were pre-treated with each CDK1i, CDK2i, CDK4/6i, Rosco, DMSO, or mock treated while under the CHX block. Four hpi, wells were washed three times with PBS and appropriate treatments were added. CDK1i, CDK2i, CDK4/6i, Rosco, DMSO, and mock treatments were all performed. Samples were collected and frozen at -80°C at 24 hpi. Samples were sonicated and spun down prior to titering using standard crystal violet plaque assays on Vero (for KOS) or L7 cells (for 7134).

3.2.6 *Western Blot Assays*

Cells were seeded in 12-well plates at a concentration of 1×10^5 cells per well. 24 hours post seeding, cells were treated with CHX and then infected with KOS at an MOI of 2 PFU/cell. 3.5 hpi, cells were treated with specific inhibitors or mock treated. Wells were then washed with PBS 3x for 4hpi. Samples were processed with laemmli as previously described at 24 hpi. Samples were ran out on NU-PAGE 8%-12% gradient gels at 130 volts for 90 minutes and then

transferred to nitrocellulose membranes. Blots were blocked with 5% BSA TBST for an hour at room temperature. Primary antibodies were used against ICP0 (mouse IgG2, Santa Cruz, 11060) at dilution of 1:1000, ICP4 (mouse monoclonal) at 1:1000, UL42 (mouse, Millipore MAB8674) 1:1000, VP5 (mouse, Santa Cruz, sc-56989) 1:1000, TK (mouse – a generous gift from Dr. William C. Summers) at 1:200, ICP8 (rabbit – a generous gift from Dr. David M. Knipe) at 1:1000, US11 (mouse – a generous gift from Dr. Ian J. Mohr) at 1:4000, actin (santa cruz rabbit, sc-1616R) at 1:1000. Antibodies were diluted in 5% BSA TBST and were incubated at room temperature for an hour. Blots were then washed 3x with TBST. Secondary antibodies used for the western blot were either goat anti-mouse horseradish peroxidase (HRP) (Jackson ImmunoResearch, 111-035-144) at 1:1000 or goat anti-rabbit HRP (Jackson ImmunoResearch, 205-035-108) at 1:1000 and were diluted with 5% BSA TBST. Membranes were washed 3x with TBST at room temperature. Membranes were developed with either West Pico chemiluminescent substrate or West Femto chemiluminescent substrate. Images of developed membranes were taken with a Kodak 400R image station.

3.2.7 *Reactivation Assays*

HFT cells were seeded in 24-well plates at a concentration of 7.5×10^4 cells per well. Cells were infected with *in1374* at an MOI of 0.3 PFU/cell for 24 hours to allow for a quiescent infection to establish. Twenty-four hpi, CHX was added to the wells to as previously described and either KOS or *d/x3.1* was

used to infect wells 1 h post CHX treatment at a MOI of 1 PFU/cell. CDK inhibitor treatments, and washes were done as previously described in section 3.2.5. Wells were then fixed at 24 hpi with 3.7% formaldehyde/PBS for 5 minutes, then washed with PBS. Wells were stained with X-gal [95] to detect β -galactosidase (β -gal) activity from reactivated cells. Cells were counted in various fields of view (3 per well) and blue cells were then compared to the total number of cells (blue/total). These values were then compared to mock-treated, KOS-infected cells that were set at 100%.

3.3 Results

3.3.1 *A high throughput screen (HTS) identified potential cellular kinases that impair ICP0's transactivation activity.*

We first set out to design a HTS to identify specific cellular kinases that were important for ICP0's transactivation activity. We used *d106* and *d109* reporter viruses (Fig. 3.1) in our HTS: *d106* reporter virus only expresses ICP0 of the five immediate early genes, while the *d109* reporter virus does not express any IE genes. Both viruses are replication-incompetent and express an HCMV major immediate early promoter driven green fluorescent protein (GFP) in place of the *UL54* locus. We established that ICP0 in the *d106*-infected cells is able to transactivate the HCMVMIEp-GFP compared to a control *d109* that doesn't express ICP0 [62]. The cell based assay was adapted to be used with an siRNA library against ~ 607 known and putative cellular kinases. We were able to identify 21 candidates that appeared to be important for ICP0's transactivation

activity based on the GFP level of the infected cells. GFP values were compared to a non-cellular kinase siRNA-treated (*GAPDH*), *d*106-infected control, which is set at 100%. We also established that siRNA targeting *GAPDH* was able to knockdown our target >95% by reverse transcription quantitative PCR and that this did not affect the GFP expression in the *d*106-infected wells. Results were considered a significant change when GFP expression was 20% or less of *GAPDH*-transfected, *d*106 infected cells. The majority of the candidates (17) were identified at 12 hpi (Table 1), fewer candidates at 18 hpi (Table 2), and the least amount of candidates identified at 24 hpi (Table 3). Candidates can be separated into different groups: CDKs, calcium/calmodulin-dependent kinases, cell morphology kinases, and signaling/metabolism kinases were identified.

3.3.2 *The protein expression of ICP0 is not notably affected by siRNA targeting CDK1, 2, 4, and 6.*

After identifying kinases that could potentially be important for the transactivation activity of ICP0, we first wanted to determine if siRNAs against specific cellular kinases affected ICP0 protein levels. We reasoned that if ICP0 protein levels were diminished in our transfected samples, there would be less ICP0 to transactivate HCMV mIEp-GFP in *d*106-infected cells, leading to false positive results. For these experiments, siRNA was used to deplete the specific cellular kinases identified from our HTS, and wells were infected with *d*106 for 12 hours. We focused on the 12 h time point because we believe it corresponds with what is occurring during early lytic infection. Western blot analysis was

done to determine if the depletion of a specific cellular kinase had an effect on ICP0 protein expression (Fig. 3.2). CDK 1, CDC2L6, 2, 3, and others did not appear to affect ICP0 protein expression, and CDK4 and 6 appeared to have a minor affect on ICP0 protein expression (Fig. 3.2A, 3.2B) compared to a *GAPDH* transfected *d106* control (G *d106*). However, the depletion of CALMK1 γ , CAMKII α , CDK7, CDK8 and others did lower the expression of ICP0 (Fig. 3.2B).

Based on these results, we decided to focus on CDKs 1, 2, 4, and 6 because the targeting each of these CDKs individually did not affect ICP0 protein expression. Also, CDKs 1 and 2 are Rosco-sensitive CDKs that were shown to affect ICP0's transactivation activity [54, 55] and are potential kinases that phosphorylate region 1 of ICP0 [39]. ICP0 can also cause a cell cycle block at the G₂/M checkpoint [59], which is a cell cycle transition where CDK1 and CDK2 are active. Additionally, CDK4 has been shown to interact with ICP0 [61] and recruit cyclin D3, which interacts with CDK4 and CDK6, to ND10 bodies [61]. We also chose to focus on CDK4 and CDK6, as they are active during the G₁/S transition of the cell cycle, a transition ICP0 can block [58, 59]. Notably, CDK4 and CDK6 have been shown to overlapping functions during the G₁/S transition [96, 97].

3.3.3 CDK inhibitors do not appreciably impair ICP0 protein expression using the CHX block and release method.

We next decided to use specific CDK inhibitors to see how impairing the kinase activity of CDKs played a role in different functions of ICP0. Before carrying out the viral replication assays, we determined optimal concentrations of the CDK1 inhibitor (CDK1i), CDK2 inhibitor (CDK2i), and the CDK4/6 inhibitor (CDK4/6i) to use by performing cell cycle analysis on PI-stained cells. Analysis was done on the PI-stained, CDKi-treated cells to determine which cell cycle phase was blocked in and whether it corresponded with the appropriate CDK inhibitor. We also determined cell viability by Trypan blue stain exclusion. Cell viability was $\geq 95\%$ for each (final) CDK inhibitor concentration chosen, and these values were comparable to mock-treated or DMSO-treated HepaRG cells (data not shown).

We used the CHX block & release technique as described in [54] to ensure that ICP0 protein levels were not affected by impairing the CDK activities. To show that the CDK inhibitors utilized in our study did not affect ICP0 protein levels, we used the CHX block & release technique (Fig. 3.3A) along with western blot assays to analyze ICP0 protein expression levels. Fig. 3.3B shows that neither the CDK inhibitors, the DMSO sample, nor Rosco appeared to appreciably impair ICP0 protein expression. A 0 hpr sample was also included to show that ICP0 protein was not being expressed at the time the CHX block was released (Fig. 3.3B).

3.3.4 *CDK1 and CDK2 inhibitors impair HSV-1 replication.*

To determine which CDKs are important for HSV-1 replication, we used the CHX block and release assay with the CDK inhibitors and Rosco as a control. When CDK1 was inhibited (CDK1i), WT HSV-1 replication was impaired by 200-fold (Fig. 3.4A). WT HSV-1 was also reduced by CDK2i, but to a lesser extent at 50-fold (Fig 3.4A). CDK4/6i also impaired WT HSV-1 replication, but only by 4-fold (Fig. 3.4A). The control, Rosco, diminished replication by 1000-fold (Fig 3.4A), as expected from results published in [56] and our DMSO control had replicated similarly to our mock-treated sample (Fig. 3.4A).

To determine whether or not these results were ICP0 specific, we also did this assay with ICP0-null virus, 7134. CDK1i reduced 7134 replication by 3-fold (Fig. 3.4B), which was not substantial. CDK2i, however, impaired 7134 replication by 195-fold (Fig 3.4B), implying the effect we saw with WT HSV-1 is not ICP0 specific. Additionally, Rosco impaired 7134 replication by 245-fold (Fig. 3.4B).

3.3.5 *CDK1 and CDK2 inhibitors reduce viral protein expression.*

Next we decided to look viral protein expression to determine if it was affected by inhibiting specific CDKs. We decided to use protein expression as an indirect readout for ICP0 transactivation activity since the transactivation activity of ICP0 was impaired by siRNAs against CDKs in HTS studies (Table 3.1). For viral protein expression, we decided to look at representative IE (ICP4), E (UL42, ICP8, and TK), and L (US11 and VP5) proteins. We found that CDK1i

reduced UL42 levels (Fig 3.5), CDK2i impaired UL42, ICP8, US11 (Fig 3.5), and VP5 levels, and Rosco impaired ICP4, UL42, US11, VP5 levels (Fig 3.5), which was expected based on previous data that Rosco inhibits transcription of HSV-1 genes [98]. Thus, the activities of CDK1 and 2 are associated with viral gene expression.

3.3.6 CDK inhibitors do not affect ICP0 localization, PML dispersion, or sp100 dispersion.

Next we decided to examine ICP0 localization as well as ND10 dispersion with the CDK inhibitors to determine if the activity of specific CDKs regulated ND10 dispersion mediated by ICP0. PML and sp100 staining were tested as they are ND10 components known to be dispersed by ICP0. Once again, we used the CHX block and release technique and fixed and stained HepaRG cells 2 hpr. The 2 hpr time point was chosen, as ICP0 has been shown to disperse/degrade ND10-associated proteins >90% by this time point [99]. In Fig. 6A, ICP0 staining was apparent throughout the nucleus. PML staining either co-localized with ICP0, was dispersed or degraded by PML, or was nuclear and punctate in the non-infected cells, as expected. The staining pattern was the same in mock-treated versus all CDK inhibitor-treated cells (Fig. 3.6A), and similar to results were obtained with Rosco-treated cells by Davido *et. al.*, 2003 [55]. We also obtained a similar result with sp100 (Fig. 3.6B). ICP0 was spread throughout the nucleus and sp100 was punctate in the non-infected

cells, which was dispersed or degraded in infected cells that expressed ICP0 (Fig. 3.6B).

3.3.7 CDK1 and CDK2 inhibitors impair ICP0-directed reactivation of viral gene expression from a quiescent infection.

We infected HFT cells with *in1374* to cause a quiescent infection as previously described [75, 93]. Specifically, *in1374* contains several mutations in the viral genome that do not allow the virus to replicate under normal conditions: it contains an ICP0 deletion, a temperature sensitive ICP4, a mutated form of VP16 that is unable to stimulate IE gene transcription, and an HCMV IEp-lacZ in the TK or *UL43* locus [75, 93, 100]. Previous publications have shown that *in1374* and similar mutated viruses can infect cells and most cells will contain viral genomes, but they will be repressed [79, 100, 101] in a manner similar to latent infection. Expression of *in1374* genomes can be derepressed by superinfecting quiescently infected cells with HSV-1 [75]. Consequently, we treated and infected cells as described in Fig 3.3A, after they were infected with *in1374* for 24 hours, to determine if the CDK inhibitors have any effect on ICP0's ability to activate viral gene expression from a repressed genome. Cells were then fixed and stained for β -galactosidase (β -gal). Cells that expressed β -gal contained reactivated virus, and random fields of view were counted for the % positive cells per each well (3 per well). All samples were compared to mock-treated, WT HSV-1 (strain KOS)-infected wells, which were assigned to be 100% reactivation. CDK1i-treated cells only had 14.7% reactivated viral gene

expression compared to mock-treated cells (Fig 3.7). CDK2i-treated cells had 3.4% reactivated viral gene expression compared mock-treated cells (Fig 3.7). CDK4/6i treated cells reactivated viral gene expression to levels near mock-treated cells, while Rosco-treated cells reactivated as nearly the same as *dlx3.1* (ICP0-null mutant) superinfected levels (Fig 3.7), demonstrating that ICP0 was required to activate viral gene expression from a repressed genome.

3.4 Discussion

With our high throughput cell based assay or HTS, we were able to identify a number of specific cellular kinases that were potential regulators of ICP0's transactivation activity (Tables 3.1-3.3). As part of our initial efforts to limit specific kinases of interest, we decided to exclude siRNA kinase targets that decreased ICP0 protein levels from our HTS (Fig. 3.2). With the remaining kinases in our siRNA screen, we decided to examine the role of CDKs 1, 2, 4, and 6 in ICP0's transactivation activity, as our data in this chapter and results from previously published reports indicated a potential association between these kinases and ICP0 [39, 54, 60, 61]. As we wanted to determine the role these kinases played in ICP0's functions and lytic infection and reactivation, we used commercially available CDK inhibitors that specific blocked the kinase activities of CDK1, CDK2, or CDK4/6. With these inhibitors, ICP0's stimulation of viral gene expression and ND10 disruption (Figs. 3.5 and 3.6), HSV-1 replication (Fig. 3.4), and ICP0-mediated stimulation of viral gene expression from a quiescent infection (Fig. 3.7) were examined. Our data showed that

CDK1i reduced UL42 protein levels (Fig. 3.5), while CDK2i decreased the expression of multiple viral proteins: UL42, ICP8, US11, and VP5 (Fig. 3.5). Rosco also impaired viral protein expression similarly to CDK2i. Immunofluorescence assay demonstrated that ICP0 localization and the dissociation of ND10 components were not affected regardless of which CDK inhibitor was tested (Fig. 3.6). Viral yield assays showed that WT HSV-1 replication was inhibited 200-fold by CDK1i (Fig. 3.4A), whereas CDK2i caused a 50-fold decrease (Fig. 3.4A). Rosco diminished WT replication by 1000-fold (Fig. 3.4A), while CDK4/6i only impaired HSV-1 replication by 4-fold. When replication of the ICP0-null mutant was examined, its yields were only impaired by CDK2i (195-fold) and Rosco (245-fold) (Fig. 3.4B). Finally, reactivation of viral gene expression from quiescently infected cells was reduced to 14.7% with CDK1i compared to the mock-treated control (100%) (Fig. 3.7). CDK2i also inhibited the induction of viral gene expression to 3.4%, and inhibition by Rosco approached 0%, which was similar to superinfection with the ICP0 null mutant, *dI*x3.1 (Fig. 3.7).

The majority of our results were obtained using inhibitors that selectively impair one or more CDKs from phosphorylating their target(s). CGP74514A (i.e., CDK1i) has been reported to be a specific CDK1 inhibitor with an IC₅₀ of 25 nM *in vitro*. Although CDK1i can inhibit other kinases, it requires a significantly higher concentration (i.e., IC₅₀ = 6.1 μM for PKCα, 125 μM for PKA, and >10 μM for EGFR) [102], and several publications have shown that CDK1i specifically inhibits the activity of CDK1 [102-104]. A report by Wei, *et. al.*, 2011, indicated

that CDK1 was specifically inhibited at 2 μ M in cell culture, which is the same concentration used in our studies. Also, we used the CDK2 Inhibitor III (i.e., CDK2i), also known as CVT-313, in our experiments with ICP0 and HSV-1. Despite its potential to inhibit other CDKs at different IC₅₀s *in vitro* (e.g., 0.5 μ M for CDK2/cyclin A and 0.5 μ M CDK2/cyclin E; 4.2 μ M for CDK1/cyclin B; 215 μ M for CDK4/cyclin D1) [105], previous publications indicate that CDK2i impaired CDK2 in cell culture at 25 μ M [106]; we used a similar concentration in our tests. While it is plausible that CDK2i could block CDK1/cyclin B activity, results from Bhattacharjee, *et al.*, 2001 suggest that this possibility is unlikely. In either case, CDK2i has a higher affinity for inhibiting CDK2 kinase activity [107]. Lastly, we treated cells with the CDK4/6 Inhibitor IV (i.e., CDK4/6i) or CINK4 at 2 μ M, and it was reported that CDK4/6i has an IC₅₀ of 1.5 μ M for CDK4 and 5.6 μ M for CDK6. Little to no inhibition has been reported for CDK4/6i against CDK1/cyclin B, CDK2/cyclin A, or CDK2/cyclin E (IC₅₀ >10 μ M) [108]. Of note, when we tested CDK4/6i at concentrations >2 μ M in cell culture, cell death was plainly visible, which is why 2 μ M was chosen for our experiments.

Our data with specific CDK inhibitors indicate that WT viral growth requires the activities of CDK1 and CDK2 for efficient replication in cell culture, where CDK2 activity is necessary for high levels of ICP0-null mutant replication. Rosco, which inhibits multiple CDKs, also diminished HSV-1 replication in our experiments, analogous to one published study [56]. Thus, our data strongly suggests that CDK1 enhances HSV-1 lytic replication in an ICP0-dependent manner, and CDK2 stimulates acute replication in a manner that is not (at least

partially) dependent on ICP0. We propose that CDK1 interacts with and phosphorylates ICP0 to regulate one or more of its activities (but not ND10 disruption) to stimulate HSV gene expression and enhance lytic replication. In support of this possibility, bioinformatics studies have shown that CDK1 is one of several potential kinases to phosphorylate ICP0 [39] and one published report showed that CDK1 can phosphorylate the N-terminal third of ICP0 *in vitro* [60], which contains Phospho-region I. Phosphorylation of ICP0 in regions I and II was also found to be required for efficient HSV-1 replication in cell culture and/or acute infection in mice [41-43], linking kinases to ICP0 functions and viral growth. Although it is plausible that CDK2 has the potential to phosphorylate ICP0 and modulate one or more of its activities, our data suggest that CDK2 is an important regulator of other steps or aspects of the HSV-1 replication cycle. As inhibiting CDK2 greatly diminished E and L protein levels, it is likely that CDK2 is important for regulating the activities of two or more IE proteins. Notably, Rosco, which inhibits both CDK1 and CDK2, impairs viral transcript accumulation and DNA replication [98, 109, 110]. However, given the parallels between Rosco and CDK2i in our experiments implies that inhibition of CDK2 is a significant contributor to Rosco's phenotypes with HSV-1 during lytic infection in cell culture.

Rosco has also been shown to inhibit explant-induced reactivation from neurons [57], demonstrating that CDK activities are important for *ex vivo* reactivation. In our study, we showed that CDK1 and CDK2 are important for reactivation of viral gene expression from quiescently infected cells. In this

regard, the aforementioned study demonstrated that latently infected neurons from mice that underwent explant-induced reactivation induced the expression of CDK2 and CDK4 [57] and not CDK1, which potentially contrasts with the results of our reactivation experiment. CDK1 could still play an important role in explant-induced reactivation for HSV-1. Specifically, CDK1 expression could be at or below the limit of detection in neurons by immunohistochemistry in the aforementioned study and its kinase activity (along with other CDKs) was not examined [57]. Also, unlike the Schang study where explant-induced reactivating neurons expressed CDK4, CDK4/6i did not impair reactivation from quiescent infection in HFT cells. Although CDK4 was not expressed in resting neurons, it was expressed in explant-induced reactivated neurons [57]. One plausible set of explanations about the differential results from our studies with *ex vivo* mouse ganglia model are the cell types (neurons vs. fibroblasts) and species (mouse vs. human) used in the model systems. Additionally, it is possible that CDK2 directs reactivation in neurons, since bioinformatics suggests it is a potential phosphorylator of phos-region 1 and 2 of ICP0 [39], and Schang, *et. al.*, 2002 [57] showed explant induced reacted neurons expressed CDK2, implying that this CDK may be important for HSV-1 reactivation. In future studies, we would like to determine if the activity of the CDKs in question are important for HSV-1 reactivation in a mouse *ex vivo* model system.

Studies with other quiescent infection-reactivation systems have shown that ICP0, specifically its RING finger, is important for reactivation from

quiescently-infected cells [111]. The RING-finger motif of ICP0 also facilitates the removal of histones and repressive chromatin marks on quiescent viral genomes [111], aiding in reactivation as latent viral DNA is known to be chromatinized [112], bound by nucleosomes [112, 113], and display very low or limited lytic gene expression [114, 115]. Though it is not known whether specific CDKs are important for regulating these functions of ICP0 during reactivation, our activation of viral gene expression from a repressed genome suggest a potential role for CDKs 1 and 2 activities through ICP0 in reactivation to modify the viral chromatin state (Fig. 3.7), which is an area of future investigation.

Our study also provides another example of how CDKs appear to be important in life cycles of other herpesviruses. In this context, Rosco inhibited the replication of varicella-zoster virus (VZV), an alphaherpesvirus related to HSV-1 and -2, both in cell culture and in mice [116, 117]. Upon VZV infection, the protein expression of CDK1 and CDK2 is increased as well as the kinase activities of CDK2 and CDK4 [118]. VZV also induced the activity of CDK2 and CDK4 in non-dividing cells, implying that these CDKs may be important for VZV replication [119]. CDK1 does play a direct role in the VZV life cycle by phosphorylating a VZV major IE protein transactivator, IE62 [120], and CDK1 is incorporated into VZV virions [120]. Rosco and cells expressing a dominant-negative CDK2 were shown to inhibit transcription and replication of the beta-herpesvirus, human cytomegalovirus (HCMV) [121, 122]. Lastly, the gammaherpesvirus, Epstein-Barr virus (EBV), requires the activities of Rosco-sensitive CDKs to reactivate from B cells [123]. Our data extend the

understanding and highlight the importance of CDKs in virus-host interactions, facilitating the switch between lytic and quiescent infections of herpesviruses.

3.5 Tables

Function/family	HTS Cellular Kinase Candidates
Cell cycle/cyclin-dependent kinases (CDK)	CDK1, CDK2, CDK3, CDK4, CDK5, CDK6, CDK7, CDK8, CDK11, CDK13
Calcium/calmodulin-dependent kinase (CALM/CAMK)	CALM3, CAMK1G, CAMK2B, CAMK2D, CAMK2A
Cell Morphology	CDC42BPA, CDC42BPB, CDC42BPG
Signaling/Metabolism	DOK1
CALM= Calmodulin, CAMK= calcium/calmodulin dependent protein kinase, CDC42 = CDC42 binding protein kinase, DOK1 – docking protein 1 62kDA	

Table 1 Cellular kinases at 12 hpi $\leq 20\%$ compared to siRNA against GAPDH *d106* control set at 100%. The screen was repeated 3 times.

Function/family	HTS Cellular Kinase Candidates
Cell cycle/cyclin-dependent kinases (CDK)	CDK1, CDK2, CDK3, CDK6, CDK11
Cell Morphology	CDC42BPA
Signaling/Metabolism	DOK1
CDC42 = CDC42 binding protein kinase, DOK1 – docking protein 1 62kDA	

Table 2 Cellular kinases at 18 hpi $\leq 20\%$ compared to siRNA against GAPDH *d106* control set at 100%. The screen was repeated 3 times.

Function/family	HTS Cellular Kinase Candidates
Cell cycle/cyclin-dependent kinases (CDK)	CDK11
Signaling/Metabolism	DOK1
DOK1 – docking protein 1 62kDA	

Table 3 Cellular kinases at 24 hpi $\leq 20\%$ compared to siRNA against GAPDH *d106* control set at 100%. The screen was repeated 3 times.

3.6 Figures and Figure legends

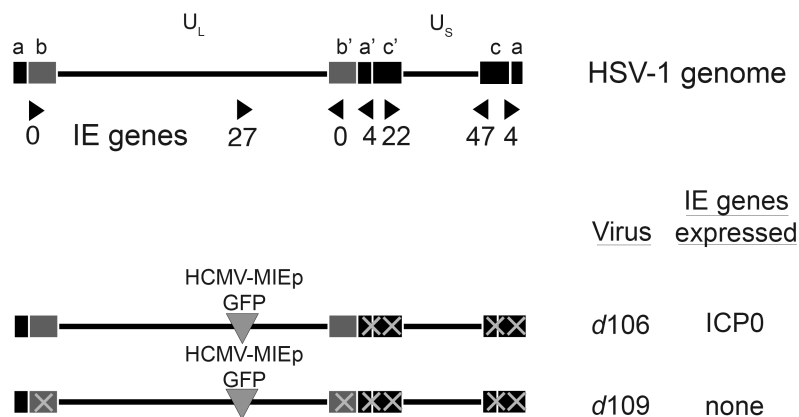


Figure 3.1 HSV-1 recombinant viruses, d106 and d109, utilized in our siRNA screen against known and putative cellular kinases.

Schematic of viruses used in the high throughput cell based assay. This figure is from Fowler, *et. al.*, 2015.

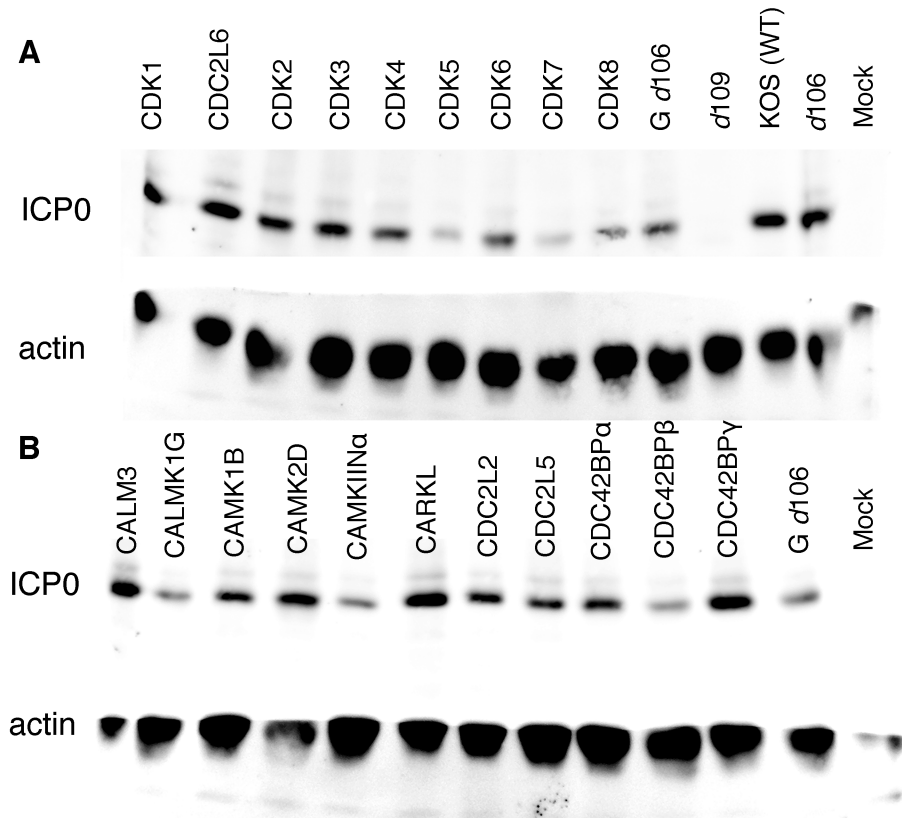


Figure 3.2 Affect of specific kinase siRNAs against on ICP0 protein levels in *d106*-infected cells.

Cells were transfected with siRNAs against specific cellular kinases in a 96-well plate and infected with *d106*. An siRNA against *GAPDH* (G *d106*) was used as a control. Samples were collected at 12 hpi and separated on SDS-PAGE, and membranes were probed for ICP0 and actin. A representative set of data from two independent experiments is shown.

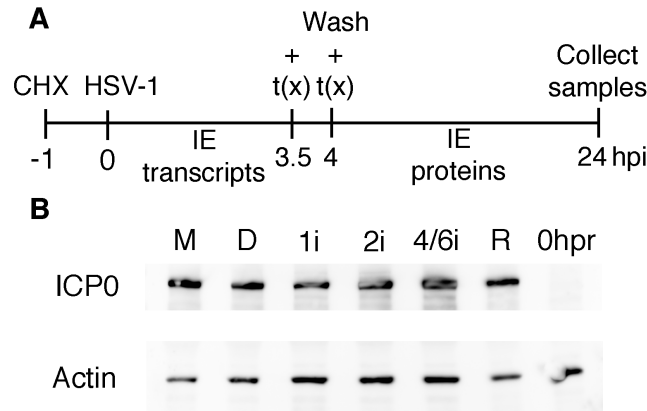


Figure 3.3 CDK inhibitors do not noticeably decrease ICP0 protein levels when added after release from a CHX block.

A) Schematic of a CHX block and release that is extensively used in subsequent experiments. CHX was added at concentration of 50 $\mu\text{g/mL}$ 24 h post seeding and 1 h prior to infection. Wells were treated with specific inhibitors 3.5 hpi. At 4 hpi cells were either harvested or released into medium with or without various treatments and samples were collected 24 hpi unless specified otherwise. B) HepaRG cells were infected with WT HSV-1 (KOS) at an MOI of 2 PFU/cell using the CHX block and release into the following inhibitors or treatment groups. Samples were analyzed for ICP0 and actin protein levels. M, Mock; D, DMSO; 1i, CDK1i; 2i, CDK2i; 4/6i, CDK4/6i; R, Rosco; 0hpr, 0 h post release. A representative set of images is shown, from 3 independent experiments that were done in duplicate.

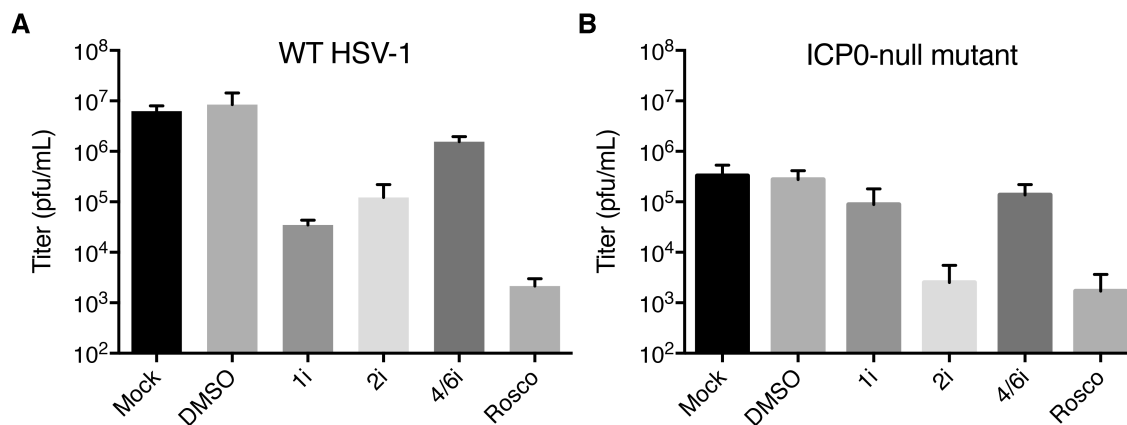


Figure 3.4 CDK inhibitors impair HSV-1 replication.

HepaRG cells were infected with KOS (WT HSV-1) (A) or 7134 (ICP0-null mutant) (B) at an MOI of 1 PFU/cell using a CHX block and release as described in Fig. 3.3A. Specific inhibitors were added 30 minutes prior to CHX release and after the CHX release. Samples were collected 24 hpi, frozen at -80°C, sonicated, and then titered out on appropriate cell line. Samples were done in triplicate and this assay was performed 3 times.

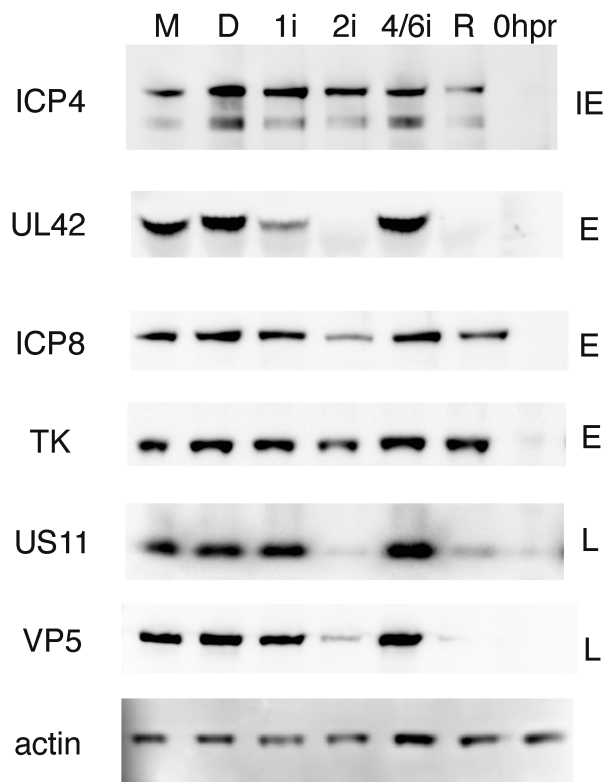


Figure 3.5 CDK inhibitors reduce the expression of a subset of HSV-1 proteins.

HepaRG cells were infected using the CHX block and release method with KOS (WT HSV-1) at an MOI of 2 PFU/cell as described in Fig. 3.3A. Samples were harvested at 24 hpi. Viral protein and actin levels were determined by western blot analyses. M, Mock; D, DMSO; 1i, CDK1i; 2i, CDK2i; 4/6i, CDK4/6i; R, Rosco; 0 hpr, 0 hours post release. A representative set of images is shown from 3 independent experiments that were done in duplicate.

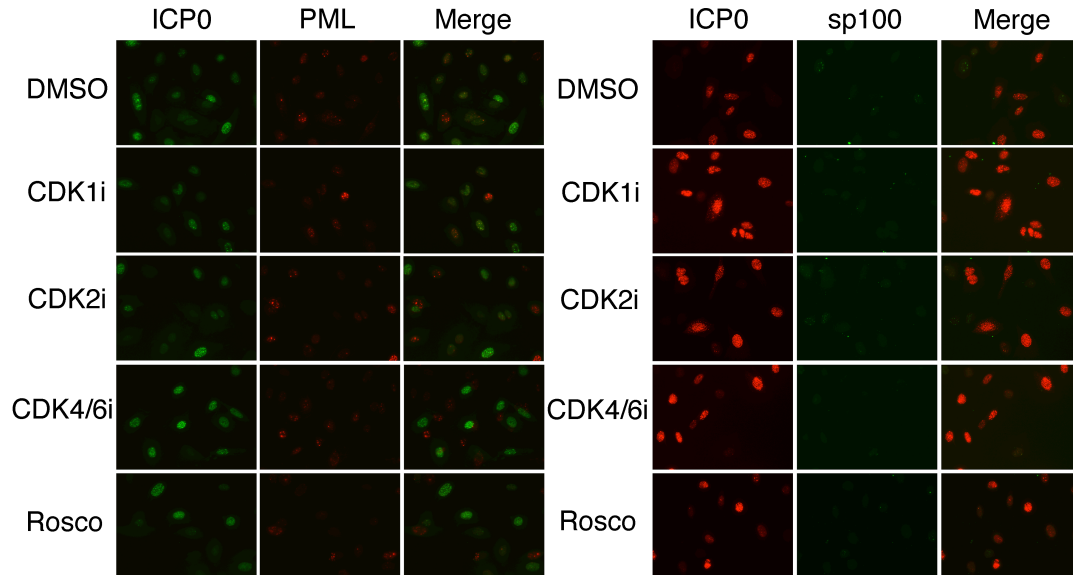


Figure 3.6 CDK inhibitors do not appear to alter ICP0 localization or disruption of ND10s.

HepaRG cells were infected with KOS (WT HSV-1) at a MOI of 2 PFU/cell using the CHX block & release in Fig 3.3A, except cells were fixed and permeabilized at 2 hpr. Immunofluorescence staining was performed to detect ICP0 and PML or ICP0 and sp100 expression. A) ICP0 (green) and PML (red) staining are in the Merge images. B) ICP0 (red) and sp100 (green) staining are in the Merge images. A representative set of images is shown from three independent that were done in triplicate.

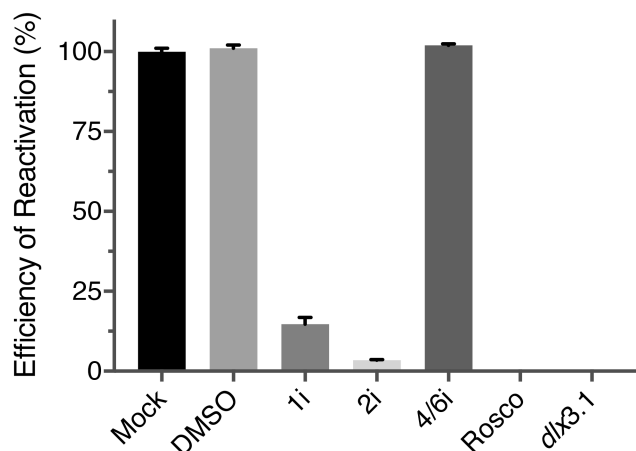


Figure 3.7 Specific CDK inhibitors diminish ICP0-directed reactivation of viral gene expression from quiescent infection.

HFT cells were quiescently infected with *in1374* at a MOI of 0.3 PFU/cell. Twenty-four hpi, cells were infected with KOS (WT HSV-1) or *dlx3.1* (ICP0-null mutant) at an MOI of 1 PFU/cell using the CHX block & release in Figure 3.3A. After 24 hpi superinfection, wells were fixed and stained for β -gal activity. β -gal positive and negative cells were counted to determine the relative % of β -gal-positive cells. The value for KOS-infected, mock-treated samples (Mock) was set as 100% for efficiency of reactivation (viral gene expression). All treatments were done in triplicate from two independent experiments.

Chapter 4: Conclusions & Future Directions

In order to begin to determine what cellular kinases are important for regulating functions of ICP0, we developed a high throughput cell-based assay (Chapter 2) that we used with an siRNA library against known and putative cellular kinases (Chapter 3). We identified a number of specific targets of which we decided to narrow down our list of potential candidates by determining which siRNA candidates siRNA did not noticeably alter ICP0 protein expression and then decided to focus our studies on CDK1, CDK2, CDK4 and CDK6. We used specific CDK inhibitors to determine that inhibiting CDK1 impairs WT HSV-1 replication while it does not impair ICP0-null mutant replication. The CDK2 inhibitor impairs both the replication of WT HSV-1 and an ICP0-null virus. Rosco also greatly reduced viral replication, while CDK4/6i did not impair HSV-1 replication. To begin to elucidate potential mechanisms of how HSV-1 replication was impaired, we next examined the expression of a subset of HSV-1 proteins. Viral protein expression was used as a readout of ICP0 transactivation activity. CDK1i decreased UL42 levels, while CDK2i, and Rosco had a much greater affect, diminishing UL42, ICP8, US11, and VP5 levels. CDK4/6i did not alter the expression of the viral proteins we examined. We next decided to see if the activities of specific CDKs were required for ND10 (PML and sp100) dispersion by ICP0 or if CDK inhibitors had an affect on ICP0 localization. None of the CDK inhibitors or the Rosco control affected ICP0 localization or it ability to disperse ND10 components. Finally, we examined

ICP0's ability to reactivate viral gene expression from a quiescent HSV-1 infection in the presence of specific CDK inhibitors. We found that CDK1i, CDK2i, and Rosco all reduced the viral gene expression from quiescent HSV-1 infection compared to WT HSV-1 viral gene expression or de-repression of the viral genes.

Based on our results in Chapter 3, we propose that CDK1 interacts with and phosphorylates ICP0 and this event is required for the transactivation activity of ICP0, which is based on our results from the siRNA screen and reduced UL42 levels, which then aids HSV-1 to achieve maximum levels of lytic infection and efficient viral gene activation from quiescent infection (Fig. 4.1). Several lines of evidence support this hypothesis. CDK1 can directly phosphorylate an N-terminal portion of ICP0 (residues 20-241) *in vitro* [60]. This portion of ICP0 contains region I (residues 224-232) of ICP0 phosphorylation that was first identified in infected cells; region I also contains potential phosphorylation sites for CDK1 or CDK2 [39]. Furthermore, the viral mutant Phos 1, which block phosphorylation sites in region I, is impaired for ICP0 transactivation activity and for viral replication in cell culture (similar to our CDK1i studies) and in a mouse model lytic replication and reactivation [39, 41-43]. Fine mapping studies of this region went on to show that serine 224 of Region I plays a major role in ICP0 activities and viral growth [43].

In future studies, we plan to determine whether ICP0 phosphorylation is directed by CDK1. We plan to employ our CHX block and release protocol infecting cell cultures with WT HSV-1 and use inducible RNAi vectors to either

deplete CDK1 or inhibit CDK1 (with CDK1i as in Chapter 3) to inactivate CDK1 during viral replication. ICP0's phosphorylation profile would be examined by tandem mass spectrometry in either depleted vs. control (for RNAi) or inhibitor-treated vs. vehicle-treated samples. To determine whether ICP0 interacts with CDK1 in cells, we propose performing co-immunoprecipitation in cell culture or (mammalian or yeast) two-hybrid assays. Should CDK1's interaction with ICP0 be transient in nature, we would use a dominant-negative version of CDK1 to stabilize this interaction. It is also possible, though less likely, that CDK1 indirectly regulates activities of ICP0.

Many publications have linked HSV to the cell cycle and CDKs. One report with the broad CDK inhibitor, Rosco, showed that it attenuates lytic replication [56], which initially suggested that the activities of CDKs are important for HSV-1 replication. Subsequent studies demonstrated that Rosco inhibited ICP0 transactivation of the HSV-1 ICP6 promoter, blocked viral DNA synthesis, and significantly reduced immediate-early and early transcript levels [54, 56, 98, 109]. Our studies with CDK2i appear to mirror many of the phenotypes observed with Rosco, strongly suggesting that the CDK2 inhibition is largely responsible for the HSV-1 phenotypes with Rosco. Overall, these data indicate that CDK activities modulate, in part through ICP0, many steps in the HSV life cycle,

While CDKs appear to regulate HSV-1, WT HSV-1 can also block the cell cycle in proliferating cells at G1/S boundary [124] or alter CDK activity [124]. Notably, HSV-1 infection can also block certain events in the G1 phase of the

cell cycle, including limiting increases in cyclins D1 and D3 levels, which are important for the G1/S transition [125]. Furthermore, infecting cells in the G1 phase with HSV-1 prevents cell cycle progression into the S phase [126]. When quiescent cells are infected with HSV-1, there is a loss of CDK4/6/cyclin D activity and an inhibition of CDK2/cyclin E activity [124]. WT HSV-1 replication in asynchronously dividing cells decreases CDK2/cyclin E and CDK2/cyclin A activity [124]. Though CDK2/cyclin E and CDK2/cyclin A activity decrease, these decreases occur later in the HSV-1 lytic infectious cycle, suggesting this decrease is an effect of viral infection. Another publication demonstrated that CDK2/cyclin A activity and expression increase early during lytic infection, but eventually decrease over time compared to serum-stimulated cells [127]. This latter study and our experiments showing CDK2i impairs HSV-1 replication, gene expression, and viral gene activation from quiescent infection implicate a pivotal role for CDK2 in the HSV-1 reproductive cycle. There is also evidence from the Roizman group that ICP0 interacts with cyclin D3 to activate CDK4 kinase activity, though it was activated to a lesser degree than in mock-infected samples [128-130]. Notably, an ICP0 cyclin D3 binding mutant has no discernable replication phenotypes in cell culture, which correlates with our CDK4/6i data, although this ICP0 mutant was less neuroinvasive in mice [129]. Thus, the activities of CDK4 and/or CDK6 during HSV-1 infection may only be apparent *in vivo*, in neurons, or both. Lastly, using a variety of HSV-1 mutants, ICP0 was shown to mediate cell cycle blocks at G1/S and G2/M [58, 59],

suggesting that the activation or inhibition of cellular factors or functions by ICP0 during these transition states may be important for HSV-1 replication.

In looking at downstream effects of CDKs on ICP0 functions, examination of its E3 ubiquitin ligase activity upon CDK inhibition is one major area of interest. ICP0 is known to degrade components of ND10s as a subset of its cellular targets. Data from our immunofluorescence experiments in Chapter 3 indicate that the activities of CDK1, CDK2, and CDK4/6 are not required for PML and sp100 dispersion, which corresponds with a previously published report [55] using the CDK inhibitor, Rosco. Outside of ND10-associated proteins, it is possible that ICP0 either mediates the ubiquitination and subsequent degradation or dispersal of other ICP0 target proteins. Relating kinase function to phosphorylation, genetic studies using ICP0 phosphorylation mutants have shown that ICP0 phosphorylation regulates its E3 ubiquitin ligase activity through protein stability by autoubiquitination and via another known target protein, USP7 [43]. In the absence of other viral factors, at least one ICP0 phosphorylation mutant form does not result in the formation polyubiquitin chains in cell culture [41].

To determine if CDKs influence ICP0's E3 ubiquitin ligase activity, we propose carrying out several experiments. The most straightforward approach would be to examine if CDK1 inhibitors impair the ability of ICP0 to form polyubiquitin chains *in vitro* or cell culture [41] or affect ICP0 stability during infection using the CHX block and release protocol. If these steps in ICP0 ubiquitination would not be affected by CDK inhibition, proteomic approaches

(the host proteome or ubiquitinome) could be performed to identify potential cellular targets whose ubiquitin status are decreased and/or stability are increased in the presence of ICP0 by blocking CDK1 activity. The identity of such targets would suggest that ICP0 differentially directs the degradation or ubiquitination of these proteins in a CDK1-dependent manner, likely impacting viral gene expression and lytic infection.

In addition to determining whether CDK1 affects ICP0's E3 ubiquitin ligase activity in cell culture, we would also like to know whether CDK1 affects ICP0 functions and HSV-1 pathogenesis *in vivo*. For these future experiments, we would use the mouse ocular model of HSV-1 infection. Specifically, we propose testing the replication of WT HSV-1, an ICP0 null mutant, and ICP0 phosphorylation mutants (e.g., Phos 1) in a WT and CDK1-knockout mouse. Such a system would allow us to examine acute infection in the eyes and neurons of the trigeminal ganglia, latency, and reactivation after explantation. We would predict that CDK1 is required for ICP0-dependent acute (peripheral and neuronal) replication in lytic infection and reactivation from latency, reflecting the results we see in Chapter 3, and a previous published study that Rosco inhibits reactivation in explant induced reactivation [57] and that ICP0 phosphorylation mutants are diminished in their ability to replicate lytically and reactivate from latency [42].

Since it appears that CDK2 enhances HSV-1 lytic replication in what appears to be an ICP0-independent manner and contributes to robust viral protein expression, we also want to begin to elucidate how CDK2 contribute to

HSV-1 productive infection. For these studies, we would inhibit CDK2 using our CHX block and release technique and infect cells with HSV-1 and perform RNA-seq to determine which viral transcripts are being expressed or impaired. Our data from Chapter 3 suggest that transcription may be impaired for at least several viral transcripts, as viral protein expression is attenuated similarly to what is seen in Rosco-treated samples. If transcription is impaired, we could also test whether or not viral DNA replication is blocked by performing real time PCR to quantitate viral genome numbers. Given the similar effects of CDK2i and Rosco in our assays and the inhibitory effects of Rosco on the HSV-1 lytic phase of infection [54, 56, 57, 109], we would predict that CDK2 participates in IE transcription, E transcription, and viral DNA replication.

It is possible that while one CDK is important for the lytic replication cycle in an ICP0-dependent manner, our data suggest that CDK1 and CDK2 may be important for reactivation. Specifically, inhibition of CDK1 or CDK2 restricts ICP0 activation of viral gene expression from a cell culture model of quiescent infection. This model system has been used to study HSV-1 latency and reactivation, given the similarities between cell-based and neuronal cultures. Specifically, the HSV-1 genome doesn't integrate into the host genome during latency, but is instead chromatinized as an episome in latently infected the neurons of mice [112] and the genomes of quiescently infected non-neuronal (human fibroblast) cells also become chromatinized [37, 113]. During neuronal latency there is limited or no lytic gene expression [131, 132], which is analogous to what happens in cell-based quiescent infections [37, 78]. These

reactivation cell culture model systems have also begun to elucidate certain factors that modulate quiescent infection and de-repression of that HSV-1 genome and how ICP0 influences these processes [37, 75, 111]. Notably, the expression of ICP0 in these models promotes the switch from repressive heterochromatin markers to transcriptional active euchromatin markers on the HSV-1 genome, which is followed by an increase in transcription.

Though there is limited information about what role CDKs and cyclins play in latency and reactivation, Schang, *et. al.*, 2002 showed that mock-infected mouse sensory neurons did not express any CDK1 or CDK2, while explant-induced reactivated neurons expressed CDK2 and CDK4 with interacting partners cyclins E and D, respectively, further supporting a role for CDK2 and ICP0 in HSV reactivation. There are several studies that have shown that CDKs are expressed in neurons when subjected to stress or death-inducing stimuli [133-139], although most CDKs that are important for cell cycle progression are not typically expressed in differentiated, post-mitotic neurons. CDKs 1, 2, 4, and 6 have also been shown to be induced and expressed during neuronal apoptosis [133, 136, 138, 140-142], a stress that can be associated with HSV reactivation. Based on the collective parallels between non-neuronal and neuronal models of quiescent infection and our own quiescent data, it is conceivable that CDK1 and/or CDK2 stimulate HSV reactivation in neurons by regulating one or more key functions of ICP0.

4.2 Figures and Figure legends

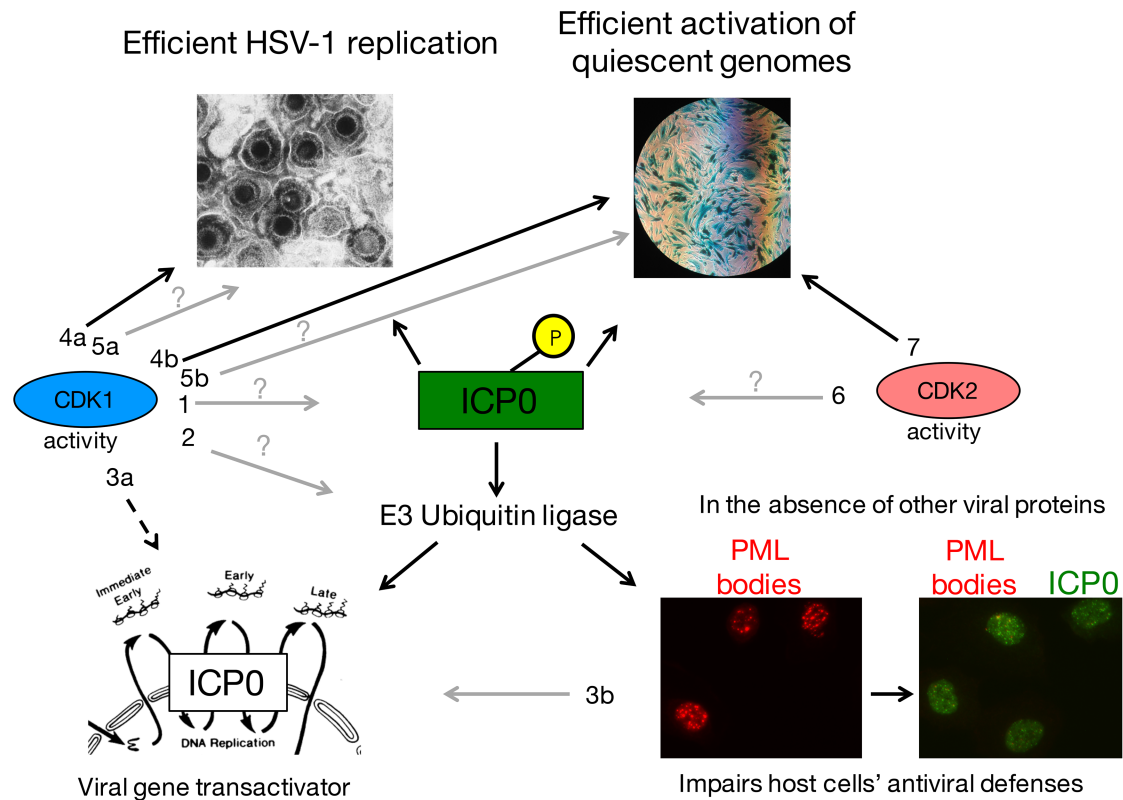


Figure 4.1 Model for how CDK1 activity regulates functions of ICP0, HSV-1 replication, and reactivation.

We propose that 1) CDK1 phosphorylates ICP0, which is 2) required for its E3 ubiquitin ligase activity. ICP0's E3 ubiquitin ligase activity and 3a) CDK2 is important ICP0 transactivation activity. The E3 ubiquitin ligase activity and phosphorylation of ICP0 is also important for dispersion of PML bodies in the absence of viral proteins [41]. 3b) The dispersion of PML bodies also aids the transactivating activity of ICP0. 4a) These activities contribute to efficient HSV-1 lytic infection and 4b) efficient activation of quiescent genomes from quiescently

HSV-1 infected cells in cell culture while 5a and 5b indicate potential relationships between CDK1 and ICP0 *in vivo* or in a mouse model. 5a) indicates lytic replication while 5b) indicates reactivation *in vivo* 6) We also propose that CDK2 potentially phosphorylates ICP0 and 7) CDK2 activity is needed for efficient activation from quiescent genomes. Black arrows indicate known steps in ICP0, while grey arrows with “?” indicate hypothetical steps or relationships. The grey arrow without the question mark is a known link or step that does not require CDK1 or CDK2. The dashed arrow indicates the relationship is based on the data from the siRNA screen and decreased UL42 levels when CDK1 activity was inhibited.

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